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Characterization of Avian Oocyte
Lipoprotein Receptors

by

Dwayne L. Barber



A Thesis

Submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

Spring, 1992



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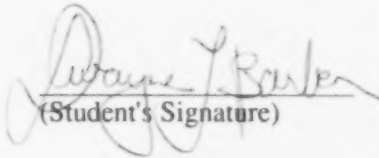
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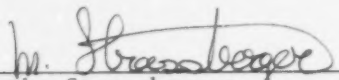
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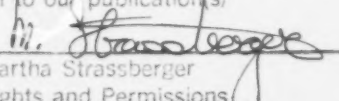


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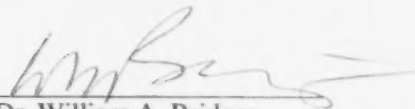
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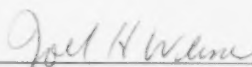
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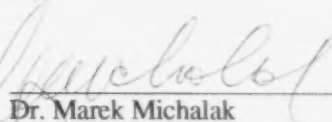
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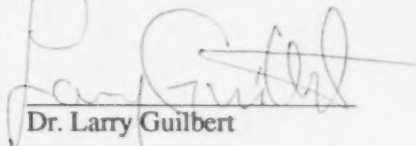
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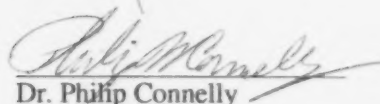
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ABSTRACT

The growth and development of the avian embryo is dependent upon the prior uptake of serum proteins into the unfertilized oocyte. This study has provided biochemical and ultrastructural evidence that a single oocyte membrane 95 kilodalton (kDa) protein functions as the receptor for both very low density lipoprotein (VLDL) and vitellogenin (VTG). The 95 kDa receptor bound low density lipoprotein (LDL) as well as mammalian apo E. VLDL, LDL and VTG all bind to the purified 95 kDa receptor in the nanomolar range expressing a single class of binding sites. Ligand competition experiments suggest that VLDL and VTG share binding sites on the 95 kDa receptor. The 95 kDa receptor is a glycoprotein that harbours both N- and O-linked carbohydrate. The receptor is clearly a member of the LDL receptor family as sequence information from three distinct regions obtained from internal tryptic peptides aligns with corresponding amino acid sequences from LDL receptors of 5 different species.

Another oocyte membrane protein was purified which displayed identical biochemical properties to the 95 kDa receptor. This 380 kDa protein bound VLDL, LDL, and VTG with high affinity in a specific and saturable fashion. Discrete ligand binding sites are present on the 380 kDa protein as suggested by competition experiments. This protein is an oocyte form of the LDL receptor-related protein (LRP) as verified by sequence comparison of internal peptides with the sequence derived from cDNA cloning of human LRP. LRP-380 was co-immunolocalized with the 95 kDa receptor to vesicular structures in the oocyte proper, illustrating that these proteins may play a mutual role in receptor mediated delivery of VLDL and VTG into the growing oocyte.

In addition, oocyte LRP-380 shares properties with its processed somatic counterpart LRP-515. Both proteins bind $^{45}\text{Ca}^{2+}$ and ^{125}I -VTG in blotting experiments. Somatic LRP-515 and oocyte LRP-380 are immunologically distinct and partition in the

aqueous phase in Triton X-114 experiments illustrating that both proteins are processed forms of a larger LRP precursor.

In summary, this research has described two new members of the LDL receptor family that function in ligand delivery in oviparous species. A 95 kDa receptor and a 380 kDa LRP transport VLDL and VTG into the growing oocyte. These proteins are related to but distinct from the somatic 130 kDa LDL receptor and the processed somatic LRP-515.

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LIST OF ABBREVIATIONS AND SYMBOLS

apo	apolipoprotein
B _{max}	maximum amount of ligand bound
BSA	bovine serum albumin
β -VLDL	β -migrating very low density lipoprotein
(c)DNA	(complementary) deoxyribonucleic acid
CHAPS	(3-((3-Cholamidopropyl)-dimethylammonio)-1-propanesulfonate)
CMC	critical micelle concentration
d	density
DEAE	diethylaminoethyl
DTT	dithiothreitol
EC	esterified cholesterol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
endo	endoglycosidase
ER	endoplasmic reticulum
FH	familial hypercholesterolemia
FSH	follicle stimulating hormone
galNAc	N-acetylgalactosamine
glcNAc	N-acetylglucosamine
gp	glycoprotein
HDL	high density lipoprotein
HDL _c	high density lipoprotein isolated from cholesterol fed animal
HDL _p	high density lipophorin
HMG	3-hydroxy-3-methylglutaryl
HPLC	high performance liquid chromatography

IgG	immunoglobulin G
IODO-GEN	1, 3, 4, 6-tetrachloro-3 α , 6 α -diphenylglycouril
kb	kilobase
kDa	kilodalton
K _d	ligand concentration which gives half maximal binding
LCAT	lecithin:cholesterol acyltransferase
LH	luteinizing hormone
LRP	LDL receptor-related protein
M _r	molecular weight
(m)RNA	(messenger) ribonucleic acid
(m)U	(milli) units
NeuNAc	N-acetylneuraminic acid
nt	nucleotide
PBS	phosphate buffered saline
PC	phosphatidylcholine
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
R/O	restricted ovulator
SDS	sodium dodecyl sulfate
UC	unesterified cholesterol
(V)LDL	(very) low density lipoprotein
VTG	vitellogenin
WHHL	Watanabe heritable hyperlipidemic
y	yolk
A	adenine
C	cytosine

G	guanine
U	uracil
A	alanine
C, cys	cysteine
D	aspartate
E	glutamate
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
X	any amino acid
Y	tyrosine

CHAPTER I
INTRODUCTION

The growth and development of the egg is central to the reproductive effort of many species. The protection of the embryo from harsh environmental conditions became a necessary requirement during the evolution of vertebrates from water to land and is thus vital for reptilian and avian survival. Therefore, the production of a cleidoic or shelled egg relies that the nutritional complement of the oocyte must be complete by ovulation and that internal fertilization occurs prior to shell formation. Given these restrictions, the avian oocyte represents a fascinating model system to study oocyte growth and protein transport.

Our research focusses on *Gallus domesticus*, the domestic hen, a well studied model of avian development. The hepatic synthesis of a number of serum proteins is under estrogen regulation in the laying hen. Furthermore, it is known that these proteins or processed counterparts are concentrated in the oocyte. It has been hypothesized that specific receptors mediate the uptake of these secreted serum proteins into the oocyte. In 1979, elegant morphological studies provided the first ultrastructural evidence that lipoprotein-like particles are transported into the avian oocyte via receptor mediated endocytosis involving the classical coated pit, coated vesicle pathway.

My studies have focussed on the isolation of a putative receptor for the triglyceride-rich, cholesterol-rich lipoprotein, very low density lipoprotein (VLDL) from avian oocyte plasma membrane. Together, VLDL and vitellogenin (VTG) account for 82% of yolk solids and 100% of yolk lipids [1]. The domestic hen was chosen as an appropriate model to study oocyte growth because of (i) the wealth of information already available about this system, (ii) its consistent egg production and (iii) its dramatic oocyte growth. In order to lay the framework for the experiments presented within this thesis, an introduction will provide the reader with background information. Pertinent data from other systems will augment the description of lipoprotein secretion and uptake in the domestic hen.

I. A. OOCYTE GROWTH

I. A. 1. OOGENESIS

During avian embryo development regression of the right ovary and oviduct occurs by day 4 resulting in only a functional left reproductive tract in hens of most species [2]. By day 8, rapid multiplication of the germ cells in the left gonad result in the formation of oogonia [3]. The number of oogonia of the chick embryo increases from 28 000 on day 9 to 680 000 on day 17 and subsequently decreases to 480 000 by hatching at day 21 [4]. Oogenesis is complete upon hatching, and the oocytes become arrested after the first meiotic division to form primary oocytes. During this time the primary oocytes accumulate ribosomes, messenger (m)RNA, protein and lipids. The DNA replicates to form two paired chromatids which are aligned in order that crossing over may occur. These chromosomes then decondense and form lateral loops which are the distinctive "lampbrush" chromosomes which are indicative of a chromosome actively engaged in RNA synthesis [5].

I. A. 2. VITELLOGENESIS

This phase is characterized by three distinct stages including a period of slow growth of the oocyte enduring over months or years, resulting in the deposition of neutral fat in the yolk. This is followed by an intermediate phase lasting 60 days in which limited amounts of yolk are added and a final rapid growth phase lasting 7 to 11 days when serum proteins are transported into the oocyte [6]. During this last phase the oocyte may increase in weight from 0.5 to 19 g. The term vitellogenesis does not strictly apply to avian species, as avian and perhaps even piscine, reptilian and amphibian oocytes have more strict lipid requirements that cannot be met merely by the uptake of a phosphoglycolipoprotein, vitellogenin. As a result in avian species, very low density lipoprotein (VLDL), in addition to vitellogenin is transported into the oocyte. Yolk precursors such as VLDL and vitellogenin are synthesized in the liver under the control of estrogen. Other hepatically synthesized precursors are also found concentrated in the oocyte. These include retinol

binding protein [7], riboflavin binding protein [8], biotin binding protein [9], transferrin [10], [11] and immunoglobulins [12], [13], [14]. It is evident that these proteins perform an essential role in the transport of vitamins and other co-factors required by the developing embryo.

At the termination of the rapid growth phase, the oocyte has one of two options: ovulation or atresia. Follicular atresia or reabsorption occurs during molting and will not be discussed further. The structure of the follicle and its contribution towards ovulation will be considered in the next section.

I. A. 3. FOLLICULOGENESIS

The avian follicle is a complex array of membranes (Figure I 1) that has been described in electron microscopy studies. During the rapid growth phase, the follicle serves to bath the surrounding oocyte in a pool of serum proteins, in addition to preserving the structural integrity of this rapidly growing cell. The follicle releases the secondary oocyte during ovulation.

The **oocyte membrane** is highly invaginated, particularly at early stages of growth [15]. The **perivitelline layer** is acellular and is believed to be produced by the granulosa cells [16], [17] reaching a final thickness of 1-3 mm. The **granulosa cells** are of somatic origin and play a role in follicular steroid production. Granulosa cell shape changes through the rapid growth phase from columnar to hexagonal to a flattened cuboidal shape [15]. The apical border of the granulosa cell has microvilli that contact the oocyte cytolemma whereas the border in contact with the basal lamina is smooth [15]. The **basal lamina** is a 1 μm thick collagenous envelope surrounding the oocyte [15]. In most species the basal laminae generally act to prevent passage of lipid. However, the avian oocyte basal lamina is thicker and appears to have lipoprotein particles embedded in it [18]. This structure may have a slightly different function in oviparous species. The theca differentiates into the **theca interna** and the **theca externa** when the oocyte reaches a diameter of 2 mm [19]. The theca interna, about 25% of the thickness of the entire theca, is

highly vascularized [20] and probably serves to supply serum proteins to the basal lamina for uptake. The theca externa forms the main mass of vesicular wall, but has not been the subject of extensive examination. It consists of elongated fibroblast-like cells [15] and also contains collagen and elastic fibres [19]. The final outer layers are the ovarian epithelium and the connective tissue covering. The **stigma** is a specialized region of the follicle through which the oocyte is released upon ovulation. Little is known about its structure, however, it is pale and relatively avascular.

I. A. 4. OVULATION

The factors that determine termination of yolk deposition in the oocyte are not well documented. In addition, it is unclear whether protein uptake occurs up to the period of ovulation or if it ceases one day prior to ovulation.

Ovulation is initiated from the concerted action of luteinizing hormone (LH) and follicle stimulating hormone (FSH) [21]. FSH action alone contributes to ovarian steroidogenesis. Hypothalamic lesions affect anterior pituitary function, subsequent LH production, and interrupt ovulation [21]. Estrogen delays ovulation, whereas testosterone causes premature ovulation [21]. Neural mechanisms have been shown to be important for ovulation, since ovulation is blocked by anti-adrenergic and anti-cholinergic drugs [21]. The role of progesterone, secreted by the granulosa cells is unclear. LH is released from the pituitary and the oocyte is released through the stigma 4-6 hr later.

At this time, the oocyte is captured by the region of the oviduct termed the infundibulum, where fertilization occurs. The infundibulum produces the first layer of albumen and transports the oocyte to the magnum where the remainder of the albumen is synthesized. The oocyte moves to the isthmus where the shell membranes are synthesized [22]. The developing egg then moves to the shell gland, where "plumping", the addition of water to the egg and calcification of the shell occurs. Once this is complete, oviposition occurs and the intact egg is expelled through the vagina [22]. The structure of the avian reproductive tract is shown in Figure I 2.

I. B. STRUCTURE AND BIOSYNTHESIS OF YOLK PRECURSORS

I. B. 1. BIOSYNTHESIS AND SECRETION OF VLDL

Lipoproteins have evolved to facilitate the transport of cholesterol, cholesteryl esters, phospholipids and triglycerides to peripheral tissue through the circulatory system. The structure of a lipoprotein particle is rather complex. Triglycerides and cholesterol esters are localized to the hydrophobic core of the particle, whereas more polar constituents such as cholesterol and phospholipids are found on the surface of the particle.

With the correlation of hyperlipoproteinemia and atherosclerosis in man, interest was renewed in conducting a detailed examination of the synthesis of VLDL. This was possible with the development of SDS-polyacrylamide gel electrophoresis to analyze individual apolipoproteins and through the use of immunological approaches. The chicken proved to be an appropriate model system since only two apolipoproteins are found on the nascent VLDL particle: apolipoprotein B and apo VLDL-II as compared to apo B, E, CI, CII and CIII on human VLDL. VLDL is the predominant circulating lipoprotein in laying hens as the conversion to LDL through lipoprotein lipase does not occur.

It was first demonstrated that plasma VLDL levels rose in a dramatic fashion during the onset of egg laying in the hen [23]. This effect was attributed to the estrogen induction of VLDL synthesis, because a similar observation was made following estrogen treatment of roosters [24].

VLDL is assembled in the liver as shown in a number of studies. Rooster liver slices were shown to have four-fold increase in VLDL synthesis in response to the addition of estrogen as determined by an immunoprecipitation assay [25]. Hepatocyte cultures from two- to three-week old chicks were the source for many experiments on avian VLDL secretion by Daniel Lane and co-workers [26]. In pulse chase-experiments, ^3H -leucine labelled, cellular apo B appeared to peak 10 min after the pulse [27]. Assuming a molecular weight of 512 000 for apo B, this corresponds to a translation rate of 7.5 amino acid residues/sec which compares well to values of 5.5-6.9 residues/sec reported for other

systems. Secreted apo B is found in the medium 30 min into the chase period, implying that 40 min are required for the synthesis, processing, assembly and secretion of the apolipoproteins of the VLDL particle [27]. N-linked carbohydrate is added co-translationally to the growing apo B chain as shown by incubation of primary hepatocyte cultures with tunicamycin. This inhibited 75-90% of ^3H -glucosamine incorporation into apo B, whereas ^3H -leucine incorporation was inhibited only 15-20% by this antibiotic [28]. Assembly and secretion of VLDL particles are independent of apo B glycosylation [29]. Hepatocytes were incubated in the presence of cycloheximide to determine the effect of apolipoprotein synthesis on VLDL secretion. Hepatocytes were pulse-labelled with ^3H -palmitate and secreted VLDL was immunoprecipitated. Under these conditions 81% of the label was recovered in the VLDL immunoprecipitate. Cycloheximide had no effect on ^3H -palmitate utilization. However, secreted levels of ^3H -labelled triglyceride and phospholipid were 26 and 25%, respectively, of incubations in the absence of cycloheximide [29]. Therefore, the availability of nascent apo B chains to assemble VLDL particles, in part, regulates VLDL secretion.

In summary, VLDL secretion can be divided into a number of contiguous phases: synthesis of apo B, core glycosylation and sequestering in the lumen of the endoplasmic reticulum (ER) which is completed within 10 minutes. Both triglyceride and phospholipid are loaded in the ER. The particle travels through the Golgi where terminal glycosylation is completed and the particle is secreted 30 min after ER sequestration [30].

I. B. 1. a. Apolipoprotein B

Partial amino acid sequence of chicken apolipoprotein (apo) B has been described [31] corresponding to the carboxy terminal 10% of the mature protein. Sequence alignment with human apo B reveals only 31% nucleotide similarity and no conservation of cysteine residues or putative sites of N-linked glycosylation found in human apo B. There are an additional 73 amino acids at the carboxy terminus of the avian protein and its mRNA is present in liver and small intestine and unlike in mammalian species, also in the kidney

[31]. However, the liver is the only tissue which demonstrates estrogen responsiveness. Apo B mRNA increases rapidly to reach a maximum level of 2 500 molecules of mRNA/cell, 24 hr after estrogen administration [31].

I. B. 1. b. Apolipoprotein VLDL-II

When the apolipoprotein components of avian VLDL were first examined by SDS-polyacrylamide gel electrophoresis, a predominant 12 kDa band was observed which migrated at 6 kDa upon reduction and alkylation [32], [33]. This protein was sequenced chemically [34] and by cDNA cloning [35]. It has been termed apo VLDL-II and consists of a 24 amino acid signal peptide and a 82 residue secreted protein. Apo VLDL-II appears during estrogen induction of oocytic precursors and is found on the VLDL particle as a disulfide-linked homodimer. Studies from our laboratory [36] have shown that apo VLDL-II acts to inhibit lipoprotein lipase, the key enzyme responsible for the conversion of VLDL to LDL. A considerable portion of the energy requirement of the developing embryo is satisfied by the transport of a triglyceride-rich lipoprotein, namely VLDL, into the oocyte. Apo VLDL-II may also play a role in the structural integrity of the VLDL particle. VLDL isolated from laying hens or estrogen-treated roosters have a mean diameter of 38 nm, whereas VLDL particles circulating in a normal rooster, devoid of apo VLDL-II, have a mean diameter of 58 nm [36].

The chicken apo VLDL-II gene has been extensively studied as a model of estrogen-induced transcription. The gene has a conserved TATA region, CAAT box, a conserved cap site and ATA box [37], as well as an avian CR1 element [38]. CR1 elements are middle repetitive DNA sequences found in avian species [39]. This 300 bp sequence is thought to be of retroviral origin and may act as a transcriptional silencer [40]. The apo VLDL-II gene contains three introns as determined by heteroduplex mapping [41].

The estrogen responsiveness of the apo VLDL-II gene appears more immediate than that observed for vitellogenin. A 0-2 hr lag was followed by a rapid accumulation of apo VLDL-II mRNA to a level of 18 000 molecules per cell [42], [43]. In another study,

the kinetics of apo VLDL-II mRNA induction show a 6 hr lag before reaching maximal levels [31]. Although both apo B and apo VLDL-II genes respond to estrogen, perhaps another signal is required for maximal induction of apo VLDL-II mRNA. The level of apo VLDL-II mRNA species dropped within 4 hr of estrogen withdrawal [42], [43]. The destabilization after hormone withdrawal was further characterized. Apo VLDL-II mRNA has a half-life of 15 hr if estrogen is withdrawn after 1 day of administration. However, extending estrogen treatment beyond 3 days results in a rapid destabilization of apo VLDL-II mRNA as the half-life decreases to 1.5 hr [44]. Degradative intermediates lacking various 3' regions were shown to occur upon estrogen withdrawal in the vicinity of the sequence GAUG which map at so-called "bulge loops", as deduced from secondary mRNA structure analysis [45]. It is thought that these sites are prone to endonuclease attack.

I. B. 2. BIOSYNTHESIS OF VITELLOGENIN

Vitellogenins are highly conserved in oviparous species. Vitellogenin genes have been studied in *Caenorhabditis elegans*, *Drosophila*, sea urchin, *Xenopus* and *Gallus gallus* [46]. Nucleotide sequences have been described for the *C. elegans* vit-5 gene [47], three *Drosophila* *yp* genes [48], the *Xenopus* vitellogenin A2 gene [49] and the chicken vitellogenin II gene [50]. In various species, a number of different VTG genes are present, ranging from one in sea urchin [51] to six in *C. elegans* [52].

Vitellogenin circulates as a dimer of 440 kDa as determined by SDS-polyacrylamide gel electrophoresis [53]. Three VTG genes have been suggested in avian species from various protein chemistry experiments [54]. All are expressed simultaneously with a molar ratio of 0.33:1.0:0.08 for VTG I, VTG II and VTG III as determined from densitometric scanning of Coomassie Blue stained SDS-polyacrylamide gels [54]. These proteins have been shown to be distinct proteins by amino acid analysis, peptide mapping, phosphorus content and immunological properties [54]. The monomeric molecular weight of VTG is 180 000 as determined from gel filtration chromatography in 7 M guanidine-HCl using a

calibration curve with human apo B as a high molecular weight marker [55]. Biosynthetic studies have shown that the processing of VTG follows a specific pathway: synthesis, glycosylation, phosphorylation followed by secretion [55].

Lipovitellins were first isolated from the egg yolk [56]. Subsequent studies showed that lipovitellins could be resolved into lipovitellin-I (110-140 kDa) and lipovitellin-II (30 kDa) [57]. Lipovitellins have been somewhat refractory to study due to their limited solubility. Nevertheless, studies by Stefano Stifani have shown that binding of VTG to its receptor is mediated through the lipovitellin moiety [58]. Unfortunately, it was not determined which lipovitellin subunit mediated binding since it was impossible to separate the lipovitellins under native conditions. It would be potentially interesting to determine if the receptor binding determinant is contained within the conserved cysteine structure of lipovitellin II.

Phosvitin was isolated from egg yolk and separated from lipovitellin on the basis of its higher solubility [59]. Later studies showed that multiple phosvitins are present [60]. The major phosvitin species is 34 kDa and a minor component of 28 kDa is also found in the yolk. cDNA cloning studies have shown that these phosvitins are products of the VTG II and VTG III genes, respectively. The major phosvitin has 123 serines in a total of 210 amino acids, found in clusters of up to 14 serines, alternated with arginine and lysine [61]. Most of these serine residues are phosphorylated since there are 116 mol of phosphorus/mol of VTG I or VTG II, whereas VTG III contains 44 mol of phosphorus/mol protein [54]. Phosvitin also contains 6.5% carbohydrate by weight [62] and two potential N-linked glycosylation sites are present in the phosvitin domain of VTG II [61]. Phosvitin does not play a role in receptor binding as was previously believed [63], as this protein is unable to compete for ^{125}I -VTG binding to oocyte receptors from *Gallus* or *Xenopus* [58], [64]. Three phosvettes, E1 (18 kDa), E2 (15 kDa), and F (13 kDa) were described after hydrophobic interaction chromatography of phosvitin [65].

The chicken VTG II gene is 20.3 kb, and contains 35 exons which encode a message of 5.7 kb. Lipovitellin-I is found at the amino terminus, lipovitellin-II is encoded at the carboxy terminus, whereas phosvitin is found from residues 1112 through 1328 [61]. The exon-intron structure is conserved between the *Xenopus* VTG A2 gene and the chicken VTG II gene [66]. There is little sequence conservation in introns between the two species but there is 40% amino acid similarity and 52% nucleotide similarity between *Xenopus* and *Gallus* VTG cDNA [66]. Two of seven putative N-linked glycosylation sites are conserved between *Xenopus* and chicken, suggesting that carbohydrate may play an essential functional role. In addition, there is a conserved cysteine motif in the lipovitellin II moiety in *C. elegans*, *Xenopus*, and chicken [67]. The phosvitin moiety is the least conserved sub-structure of the VTG gene. Exon 23 of the VTG II gene encodes 203 of the 210 amino acids of phosvitin which appears to have evolved as a result of duplication of short serine stretches. A phosvitin-encoding domain has been isolated from the chicken VTG III gene, which reveals a smaller exon 23 consisting of 321 bp coding for 28 ser compared to 124 ser in the 690 bp exon 23 from the chicken VTG II gene [68]. Phosvitin was not found in insect VTG genes, suggesting that this highly negatively charged Ca^{2+} -binding protein [69] may play a role in sequestering Ca^{2+} for bone development in vertebrate species [67].

The VTG II gene is under estrogen regulation [42], displays an estrogen memory effect [43] and has similar upstream regulatory elements as the apo VLDL-II gene described earlier [70]. Upon estrogen treatment, VTG II mRNA is expressed after a 4-8 hr lag [42], [43]. However, no lag in mRNA synthesis is observed with a second administration of the hormone. Other studies have shown that the effects on VTG II transcription are manifested not only through estrogen receptors and phosphorylation of nuclear proteins alone but require other as yet unidentified, additional factors [71].

I. B. 3. BIOSYNTHESIS OF OTHER YOLK PRECURSORS

A number of other serum proteins are concentrated in the yolk. These include retinol binding protein [7], riboflavin binding protein [8], biotin binding protein [9], transferrin [10] and immunoglobulin G [72]. cDNAs have been isolated for riboflavin binding protein [73] and transferrin [11]. Genes for retinol binding protein, riboflavin binding protein [74] and transferrin [11] are estrogen-responsive, suggesting a common mode of regulation of the hepatic expression of yolk precursors.

I. C. AVIAN LIPOPROTEIN STRUCTURE AND COMPOSITION

Lipoproteins are synthesized in higher organisms in order to facilitate transport of lipids. In insects, diglyceride is transported via high density lipophorin (HDL_p) to be utilized by the organism as an energy source. In mammalian species, cholesterol is transported in low density lipoprotein (LDL) and dietary fatty acid is carried to peripheral tissues via chylomicron remnants. In oviparous species such as birds, the energy requirement of the embryo is high. The hen synthesizes very low density lipoprotein which subsequently is concentrated in the oocyte. Each lipoprotein class has a distinctive lipid composition and specific apolipoprotein constituents that mediate receptor binding as well as activation or inhibition of associating enzymes.

I. C. 1. VLDL

The biosynthesis and assembly of VLDL occurs predominately in the liver [75]. Chylomicrons are absent in birds; however, large VLDL particles (60 nm) are assembled in the intestine and enter the circulation through the portal vein as so-called "portomicrons" to transport dietary fat [76]. The lipoprotein profile of roosters and immature hens is similar, characterized by low levels of VLDL and higher levels of HDL [77]. As shown in Table I, upon the onset of laying, HDL levels fall two- to three-fold and VLDL rises dramatically. LDL concentrations are two-fold higher in laying hens. These reported values have been corroborated by other studies [32] [78].

Table I 2 illustrates that avian VLDL particles ($d = 1.006$) have higher triglyceride, phospholipid and protein content, but lower cholesterol levels than those found in human VLDL [79]. Laying hen VLDL has elevated triglyceride and phospholipid content than VLDL isolated from immature hens or roosters.

I. C. 2. LDL

Chicken LDL (23.4 nm) ($d = 1.019-1.063$) is a slightly larger particle than human LDL (21.7 nm), probably due to increased triglyceride content as shown in Table I 3 [78]. Levels of phospholipid are higher in LDL isolated from both sexes of bird, whereas like VLDL, the free and esterified cholesterol concentrations are lower in avian LDL particles than human LDL [79]. Chicken LDL has apo B as its sole apolipoprotein component.

I. C. 3. HDL

Chicken HDL ($d = 1.15 - 1.21$) has a similar composition to human HDL as shown in Table IV [79] and is characterized by lower triglyceride and free cholesterol levels than VLDL or LDL. The ratio of esterified to free cholesterol in avian HDL (EC/UC; 4-6:1) is greater than in reptiles or fish and is closer to that found in human HDL [79]. In the laying hen, lower levels of cholesterol, cholesteryl ester and phospholipid are found whereas the the protein content has increased markedly. Chicken HDL contains primarily apolipoprotein AI, a 28 kDa protein. The sequence of apo AI has been determined by sequencing of peptide fragments [80] and through cDNA cloning [81]. Its inferred size calculated from cDNA sequence is 26 674; it migrates at 28 kDa in SDS-polyacrylamide gel electrophoresis experiments.

Human and avian lipoprotein structure and composition are similar in many respects. However, in mature females, the lipid requirements of the developing embryo dictate that VLDL must be transported into the oocyte. The presence of apoVLDL-II on the VLDL particle appears to facilitate this process through inhibition of lipoprotein lipase as discussed above.

TABLE I 1
LIPOPROTEIN COMPOSITION IN WHITE LEGHORN CHICKEN [77]

	Rooster*	Non-Laying Hen*	Laying Hen*
VLDL	54	71	1401
LDL	86	83	152
HDL	370	361	151

* units expressed as mg/dl

TABLE I 2
VLDL COMPOSITION IN AVIAN AND HUMAN SPECIES [79]

	Rooster*	Non-laying Hen*	Laying Hen*	Human*
Esterified Cholesterol				14.9
	{ 9.3	{ 11.4	{ 5.1	
Free Cholesterol				6.7
Triglyceride	57.4	55.7	56.5	49.9
Phospholipid	18.5	18.6	25.8	18.6
Protein	14.8	14.3	12.7	7.7

* units expressed as % by weight

TABLE I 3
LDL COMPOSITION IN AVIAN AND HUMAN SPECIES [79]

	Rooster*	Immature Hen*	Laying Hen*	Human*
Esterified Cholesterol			7.5	38.0
	{ 20.5	{ 21.0		
Free Cholesterol			10.9	9.0
Triglyceride	30.1	19.9	36.9	11.2
Phospholipid	31.3	40.3	23.7	22.1
Protein	18.1	18.8	21.0	20.9

* units expressed as % by weight

TABLE I 4 -
HDL COMPOSITION IN AVIAN AND HUMAN SPECIES [79]

	Immature Rooster*	Rooster*	Laying Hen*	Human
				HDL ₂ (1.063-1.125)* HDL ₃ (1.125-1.21)*
Esterified Cholesterol	15.0	16.0	7.9	16.2 11.7
Free Cholesterol	2.9	5.0	1.4	5.4 2.9
Triglyceride	2.0	6.4	8.2	4.5 4.1
Phospholipid	24.0	28.7	17.6	29.5 22.5
Protein	56.1	43.9	64.0	41 55

* units expressed as % by weight

I. D. MAMMALIAN LIPOPROTEIN METABOLISM

Mammalian lipid transport is accomplished through an exogenous pathway of dietary origin and a biosynthetic or endogenous pathway.

Chylomicrons are triglyceride-rich lipoprotein particles that are assembled in the intestine and serve to transport triglyceride-derived fatty acids to peripheral tissues. Through the action of lipoprotein lipase located in the capillary, fatty acids are liberated from the chylomicron particle, resulting in the conversion of chylomicrons to chylomicron remnants. These cholesterol-rich particles contain apo E and apo B-48, but are thought to lose apo Cs, regulators of lipoprotein lipase activity [82] during lipolysis. Chylomicron remnants are transported to the liver, where it is hypothesized that a specific apo E receptor mediates their uptake. The LDL receptor binds both apo E and apo B containing particles, but its role in chylomicron remnant uptake has not been identified. Watanabe heritable hyperlipidemic (WHHL) rabbits lack functional LDL receptors but display normal clearance of chylomicron remnants [83], [84]. Therefore, it is felt that a specific receptor could mediate chylomicron remnant uptake.

VLDL is primarily synthesized in the liver and contains apo B-100, E and C. Lipoprotein lipase catalyzes the conversion of VLDL to LDL by hydrolysis of triglycerides. LDL particles are cholesterol-rich and lack apo E. LDL is removed from the circulation by the 130 kDa LDL receptor expressed in extrahepatic tissues or in the liver. The concentration of LDL in the circulation is primarily regulated by LDL receptor number, rate of hepatic VLDL synthesis and lipoprotein lipase activity.

Cholesterol and cholesteryl esters are thought to be transported from peripheral tissues to the liver by a process termed reverse cholesterol transport [85]. Hepatically derived nascent HDL particles interact with peripheral tissues and the particles load cellular cholesterol. Lecithin:cholesterol acyl transferase (LCAT) activity promotes formation of esterified cholesterol in the HDL particle. The cholesteryl ester enriched HDL then delivers cholesterol to the liver by direct interaction with liver receptors or indirectly by

transfer of cholesteryl ester to apo B-containing lipoproteins via lipid transfer protein [86], [87], [88], [89]. A specific HDL receptor has been suggested in the literature. A 110 kDa protein has been identified from ligand blotting experiments [90], but radiation inactivation experiments suggest that a 16 kDa fibroblast protein is the HDL receptor [91]. However, the existence of receptor-mediated HDL transport is still a subject of discussion [92].

I. D. 1. APOLIPOPROTEIN B

Apo B-100 is synthesized by the liver and its secretion into the lumen of the endoplasmic reticulum is thought to be essential for the proper assembly of the VLDL particle. Studies on apo B were hindered for many years due to the refractory properties of this protein. It is only sparingly soluble in high concentrations of detergents; delipidation appears to induce aggregation, and it is extremely sensitive to proteolysis.

The nucleotide sequence of apo B has been deduced from cDNA cloning [93], [94], [95], [96], [97], [98]. The extremely large apo B mRNA of 14 kb [99] encodes a protein of 4563 amino acids. Thirteen of 20 potential sites for N-linked glycosylation are utilized [98] and 8-10% of the mass of apo B is N-linked carbohydrate [100]. Apo B has a 27 residue signal peptide which is cleaved during its secretion. Secondary structure analysis reveals that apo B contains 43% α helical, 21% β -sheet, 16% β -turn and 20% random structures [98] which concurs well with previous circular dichroism studies [101]. A number of long repeats, albeit with a relatively low degree of sequence similarity (12-21%) suggest that apo B may have arisen from internal duplication.

Another form of apo B, termed apo B-48, is synthesized in the intestine and is a component of chylomicrons and chylomicron remnants. Apo B-48 is 250 kDa (48% of apo B-100) [102] and is unable to bind to the LDL receptor [103]. Immunological and protein chemical experiments suggest that the amino terminal portion of apo B-100 and apo B-48 are identical [104], [105]. Studies from three laboratories demonstrated that apo B-48 was generated by mRNA editing of CAA to UAA (a termination codon) at residue 2153 [106], [107], [108]. Apo B cDNA fragments were edited when expressed in

cells that produce apo B-48 such as CaCo-2, a human colon adenocarcinoma; in apo B-100 secreting cells, such as human hepatoblastoma, Hep G-2; and G-292, a human osteosarcoma cell line that does not synthesize either apo B-100 or apo B-48 [109]. This suggests that mRNA editing may be a general biological phenomenon. The sequence in the vicinity of the edited C at position 6666 is highly conserved as no nucleotide changes are observed from 6662 to 6684 in human, rabbit, rat and mouse apo B DNA [110]. An 11 nucleotide sequence extending from 6671 to 6681 was essential for mRNA editing as determined from primer extension analysis [111]. This sequence may be responsible for binding the editing activity which can be isolated from rat McArdle 7777 cells [112]. ^{32}P -uridine monophosphate was produced from ^{32}P -cytidine triphosphate-labelled mRNA after incubation with the editing extract, hydrolysis of the mRNA and analysis of products via thin layer chromatography [113]. This implies that endonucleolytic excision and repair does not occur; rather, an as yet unidentified cytidine deaminase is responsible for editing of apo B. Editing of C to U at position 6666 appears to activate a cryptic polyadenylation site within the apo B sequence downstream from the edited base [114]. Position 6666 is in a loop at the end of an extensive stem when the secondary structure of the sequence is analyzed [114], [111].

The amino terminus of apo B is not considered as a potential receptor binding site(s), since apo B-48 is unable to bind to the LDL receptor from fibroblasts [103]. At least three different models of apo B binding have been proposed. A single linear sequence may be involved, or a number of basic residues throughout the apo B sequence may result in a single tertiary folded binding site, or multiple binding sites may exist. The receptor binding domain of apo B was first localized using a combination of biochemical and immunological experiments [115] and was later verified by localization of epitopes by bacterial expression cloning [116].

In order to determine which areas of apo B are responsible for receptor binding, proteolytic digestion and reconstitution of apo B fragments was completed. Thrombolytic

fragments of human apo B were prepared, delipidated, resolved by SDS-polyacrylamide gel electrophoresis, electroeluted and individual fragments were iodinated [117]. Recombinant HDL_C-apo B-100 fragments were prepared by incubation with canine HDL_C that had undergone reductive methylation and trypsinization to eliminate apo E binding sites. These reconstituted fragments showed the hallmark properties of apo B interaction, namely specific binding, inhibition of binding by anti-LDL receptor antibodies and Ca²⁺-dependent binding. However, all fragments were capable of binding to the receptor and no selectivity was observed. This might not be surprising due to the large number of manipulations used to generate the individual recombinant fragments. It is unknown if the surface properties of trypsinized canine HDL_C correlate with those of human LDL. Nevertheless, these data may be suggestive of a model of LDL binding that favours multiple binding sites.

Earlier studies provided evidence that multiple binding sites are doubtful, since a single monoclonal antibody (with a binding stoichiometry of 1:1) can block LDL receptor binding [118], [119], [120]. A panel of 40 monoclonal antibodies directed against apo B-100 was available. The epitopes were localized by assignment of CNBr fragments or binding to bacterially expressed protein from apo B cDNA fragments [116]. Seventeen different determinants were identified and these antibodies were tested for their ability to block ¹²⁵I-LDL binding to the LDL receptor from human skin fibroblasts. Antibodies corresponding to a region near the T2/T3 thrombin cleavage site blocked ¹²⁵I-LDL binding [115]. Thrombin sensitivity of this region of apo B would suggest that this domain is exposed. Indeed, sequence analysis reveals that two clusters of basic amino acids are found in this region (**KAQYYKNKHRH**) AND (**TTRLTRKRGLK**), corresponding to residues 3147-3157 and 3359-3367, respectively [96]. A disulphide linkage between C3167 and C3297 may bring these regions in close proximity [96].

I. D. 2. APOLIPOPROTEIN E

Apolipoprotein E is involved in lipoprotein secretion and transport and may also play a role in nerve regeneration [121]. Apo E contains 60% α -helical structure with amphipathic structure [122]. It freely exchanges between lipoprotein fractions and can be found on chylomicrons, chylomicron remnants, VLDL and HDL particles. Apo E-containing lipoproteins bind to the LDL receptor with a 10- to 100-fold higher affinity than apo B-containing lipoproteins [123]. An excellent review summarizes much of our current knowledge of this protein [121].

Apo E is synthesized in many different tissues including liver, brain, spleen, lung, adrenal, ovary, kidney and muscle [121]. The protein sequence of apo E was first described in 1982 [124], followed by isolation of the cDNA one year later [125]. The isolated mRNA is 1163 bp in length [126], [127] giving a translation product of 317 amino acids containing an 18 amino acid terminal signal peptide. Secreted apo E is a 34 kDa protein.

Apo E consists of three different isoforms identified via isoelectric focussing [128], [129] and two-dimensional electrophoresis [130], termed E2, E3, and E4. The most common phenotype is apo E3 which displays normal binding of apo E to LDL receptors [121]. Apo E2 and apo E4 are considered to be variants; apo E2 is defective in receptor binding whereas apo E4 is correlated with elevated plasma cholesterol and LDL despite displaying normal binding [121]. Apo E2 has a R to C substitution at amino acid position 158, while apo E4 is characterized by a C to R substitution at amino acid position 112 [124], [131], [132].

Reductive methylation of basic residues blocked the binding of apo E to the LDL receptor [133], [134]. A large number of basic residues are located in the region of residues 140 to 160 in apo E. An amino terminal thrombolytic apo E fragment (residues 1-191) and a cyanogen bromide fragment (residues 126-218) both retained full receptor binding activity [135]. A monoclonal antibody was generated that blocked receptor binding

to apo E was epitope mapped to residues 140 to 150 of apo E (HLRKLRLRLR) [136].

I. E. THE LOW DENSITY LIPOPROTEIN RECEPTOR FAMILY

The paradigm for studies on membrane receptors and receptor mediated endocytosis is the LDL receptor. The presence of a specific receptor for cholesterol transport was first suggested in 1976, when it was shown that fibroblasts isolated from a patient suffering from familial hypercholesterolemia (FH) have the following atypical phenotypic features of normal LDL metabolism: basal levels of ^{125}I -LDL binding and uptake, low levels of apo B and cholesterol ester hydrolysis, low degree of cholesterol esterification and lack of suppression of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, a key enzyme in the cholesterol biosynthetic pathway [137]. These observations were central to the eventual protein purification [138], isolation of the cDNA sequence [139] and genomic structure of the LDL receptor [140]. This section will focus on the domain structure of the receptor and genetic analysis of FH patients as well as mutagenesis studies of the gene that have provided further insight towards understanding the functional properties of this receptor. The LDL receptor has been described extensively in two recent review articles [141], [142].

I. E. 1. THE LOW DENSITY LIPOPROTEIN RECEPTOR

The LDL receptor was purified from bovine adrenal membranes by ligand affinity chromatography and immunoaffinity chromatography and was shown to migrate on SDS-polyacrylamide gel electrophoresis as a 160 kDa integral membrane protein. Biosynthetic studies illustrated that the protein was synthesized as a precursor containing high mannose forms of N-linked carbohydrate in the endoplasmic reticulum and was sensitive to treatment with endoglycosidase H [143]. The LDL receptor precursor undergoes a dramatic shift from 120 to 160 kDa on SDS-polyacrylamide gel electrophoresis between 30 and 60 minutes after synthesis [143]. This coincides with maturation of the N- and O-linked carbohydrate chains by their movement through the Golgi, demonstrated by acquisition of endoglycosidase H resistance as well as sensitivity

to sequential treatment with neuraminidase and O-glycanase [144]. The deduced protein sequence suggests five potential sites for N-linked glycosylation, however, carbohydrate analysis has revealed that only two of these sites are utilized [145]. O-linked carbohydrate chains are also present as a pronase fragment contains multiple GalNAc residues from ^3H -glucosamine labelled epidermal carcinoma A-431 cells [145]. The 40 kDa mobility shift of the receptor on processing is not due to the maturation of N-linked carbohydrate alone, since a similar shift is observed in the presence of tunicamycin, an inhibitor of N-linked glycosylation. No shift is observed in a CHO mutant cell line that is unable to add galactose to the core GalNAc residues of the O-linked chains [145].

The LDL receptor is composed of five domains, illustrative of a molecule that must perform many functions including ligand binding, localization to coated pits, internalization and recycling to the cell surface. It is a Type I membrane protein as shown in Figure I 3 with a 21 amino acid signal sequence that is co-translationally cleaved. The amino terminal domain contains a cys-rich repeat structure which is responsible for apo B and apo E binding. The second domain contains homology with the epidermal growth factor (EGF) precursor region. An O-linked carbohydrate domain occurs next followed by a 22 amino acid transmembrane region. The carboxy-terminal 50 amino acids reside in the cytoplasmic domain. A more detailed analysis of each domain is presented in the next section.

I. E. 1. a. Ligand Binding Domain

This domain consists of 292 amino acids in seven repeats with a highly conserved cysteine-rich repeat structure. This sequence is similar to complement component 9 [146]. In fact, cysteine repeat structures have been observed in many other proteins that act as receptors such as members of the cytokine receptor family [147], immunoglobulins [148] and DNA-binding proteins [149]. A cluster of negatively charged amino acids (SDE) is found in each repeat and has been shown through site-specific mutagenesis to be important for ligand binding [150]. The hypothesis that this region plays a role in ligand binding was suggested by a number of experimental observations. Binding of the ligands apo B and

apo E can be blocked by reductive methylation of lysine residues [134]; both apo B and apo E bind strongly to heparin suggesting the presence of exposed clusters of basic residues; the interaction of ^{125}I -LDL with the receptor can be blocked by the negatively charged compound, suramin [138]; ligand binding is abolished by reduction of the receptor protein [151] and a 60 kDa amino terminal thrombolytic fragment binds ^{125}I -LDL [144].

This biochemical evidence was further supported by site-specific and deletion mutagenesis of LDL receptor cDNA. Deletion analysis of this domain revealed that repeats 2-7 are required for maximal binding of ^{125}I -LDL whereas only repeat 5 is required for ^{125}I - β -VLDL binding [152], which is thought to be mediated by apo E. The importance of acidic amino acids in LDL binding was shown by the substitution of the conserved aspartate to tyrosine in repeats 2 through 7. This altered ^{125}I -LDL binding from 8-74% of control values in COS cells expressing the receptor constructs [150].

Naturally occurring mutations in this region support results from mutagenic analysis. Fibroblasts from a FH patient have been characterized that lack exon 5, coding for the sixth ligand binding repeat [153]. This person displays clinical homozygous FH; the patient's fibroblasts are unable to bind ^{125}I -LDL, but show ^{125}I - β -VLDL binding. This mutation arose through homologous recombination between *Alu* sequences, a middle repetitive DNA sequence, in introns 4 and 5, resulting in exon 4 being adjacent to exon 6.

Fibroblasts from another FH patient harboured a 3 bp deletion in repeat 4. A 12 nucleotide deletion was discovered in the third cysteine rich repeat of the cDNA from another FH homozygote. Both of these mutations give rise to a receptor that displayed slow processing [154]. Perhaps the cell discriminates against improperly folded structures, preventing localization to the cell surface.

I. E. 1. b. EGF Precursor Homology Domain

The next domain is rather large, consisting of ~400 amino acids and bears similarity to the EGF precursor, a membrane-bound precursor form of mature EGF. Three repeat structures of 40 amino acids with six cysteine residues each, spaced at regular intervals are

found in this domain with 35% amino acid similarity observed within the repeats [139]. These repeats show similarity to other vitamin K dependent proteins such as factors VII [155], IX, X [156], protein C [157] and protein Z [158]. All of these proteins are β -hydroxylated on aspartate or asparagine residues as is the LDL receptor on D310 and N349 [159]. A tetrapeptide YWTD is also repeated five times in the LDL receptor.

This modification must be essential for LDL binding as a mutant cDNA with both β -hydroxylated residues substituted (D to A at position 310 and N to A at position 349) displays 23% ^{125}I -LDL binding of normal LDL receptor cDNA. Deletion of the A and B repeats shows 18% ^{125}I -LDL binding at saturation, whereas the binding of apo E is unaffected by deletion or substitution as shown by ^{125}I - β -VLDL binding to transfected COS monolayers [152].

Deletion of the entire EGF precursor homology domain affected acid-dependent dissociation of ligand from the receptor and subsequent recycling, resulting in rapid degradation of the receptor [160]. However, deletion of one-half of the protein sequence may have a profound effect on the tertiary structure of the resulting product. FH 381 has been characterized to have a 5 kb deletion resulting from an *Alu* recombination in intron 15 and in the 3' untranslated region of exon 18 giving rise to a secreted form of the receptor [161].

I. E. 1. c. O-linked carbohydrate domain

This domain is relatively small, but 18 serine or threonine residues are found in a 58 amino acid stretch. Glycosylation of most or all of the hydroxylated residues is thought to account for the 40 kDa shift on processing of the receptor [145]. Deletion of the O-linked domain had little effect on receptor function when expressed in transfected human fibroblasts [160]. A mutant CHO cell line unable to synthesize O-linked sugars was utilized to show that the absence of O-linked carbohydrate led to rapid turnover of the LDL receptor [162], [163].

I. E. 1. d. Transmembrane domain

The LDL receptor has a single transmembrane region of 22 amino acids; it is the least conserved of all domains.

I. E. 1. e. Cytoplasmic domain

This domain consists of 50 amino acids and is vital for receptor localization to coated pits as a number of FH homozygotes have been identified who display normal LDL binding to fibroblasts but defective internalization. One FH patient has a single base change resulting in the substitution of a cysteine for tyrosine at position 807. It was shown through *in vitro* mutagenesis that the first 22 amino acids of the cytoplasmic tail were sufficient for rapid internalization [164]. Site specific mutagenesis of tyrosine 807 showed that aromatic residues (Y, F, or W) would allow internalization to occur. There also appeared to be an absolute requirement for proline at position 805. These studies were extended to show that the sequence **NPVY** was conserved in the cytoplasmic region of all six LDL receptors with known sequence [165]. Upon examination of other receptors that are known to be localized in coated pits, it was discovered that the sequence **NPXY** was found in the insulin receptor, EGF receptor, and fibronectin receptor, amongst others. Oligonucleotide-directed mutagenesis of the LDL receptor cDNA showed that substitution of A for N804, P805, or Y 807 dramatically reduced internalization.

The importance of the cytoplasmic domain in receptor internalization was demonstrated by characterization of the LDL receptor cDNA from fibroblasts cultured from other FH patients. FH 683 has a G to A point mutation resulting in a premature termination codon giving a protein with only a two amino acid cytoplasmic tail. The internalization defective phenotype was also observed in FH 763 in which a frameshift mutation gave rise to a different reading frame. The cytoplasmic domain of this mutant receptor contains 6 amino acids of normal sequence and 8 amino acids of mutant sequence before a nonsense codon is reached. In all these FH patients, fibroblasts display normal LDL binding, but

internalization is hampered due to the absence of the essential internalization sequence NPVY.

I. E. 2. LDL RECEPTOR-RELATED PROTEIN

In 1988 another member of the LDL receptor family, LDL receptor-related protein (LRP) was described [166]. A murine lymphocyte cDNA library was screened with an oligonucleotide probe corresponding to the cysteine-rich binding repeat structure found in the ligand binding domain of the LDL receptor and complement C9. A partial clone was isolated which contained 8 repeat motifs similar to the LDL receptor. This clone was used as a probe to screen a human liver cDNA library and a 15 kb cDNA was isolated [166]. The domain map of LRP, the LDL receptor, and the EGF precursor are shown in Figure 14.

The LDL receptor contains 7 cysteine-rich repeats (designated A-type repeats) and 3 EGF precursor or B type repeats. LRP appears to have undergone a cassette duplication giving 4 large clusters of A-B repeats consisting of 31 A-type repeats and 22 B-type repeats. A large number of YWTD repeats is also present. No O-linked carbohydrate region is present in LRP. A single transmembrane spanning sequence is followed by a cytoplasmic domain that is longer than that in the LDL receptor and contains two repeats of the internalization sequence NPXY. LRP is a large protein of 600 kDa that is post-translationally processed at the sequence RHRR to generate an extracytoplasmic protein LRP-515 and LRP-85 which contains repeats of the EGF precursor region, transmembrane and cytoplasmic domains [167].

The structural features of LRP suggest that it might function as a lipoprotein receptor. WHHL rabbits lack functional LDL receptors due to a 12 nucleotide in-frame deletion [154]. These rabbits clear LDL at a slow rate but display normal clearance of chylomicron remnants [83]. It was thought that LRP may function as an apo E-specific remnant receptor. However, the evidence accumulated to date is only suggestive, but not conclusive in support of this hypothesis. FH fibroblasts bind β -VLDL particles, but only

after enrichment with recombinant apo E [168]. The conversion of ^{14}C -oleate to cholesteryl ^{14}C -oleate was shown to be dependent on the amount of apo E-enriched β -VLDL that was added to the fibroblasts. Apo C-I was an effective competitor for apo E binding to β -VLDL; the resulting β -VLDL particle is unable to bind to LRP, but still binds to the LDL receptor on ligand blots [169], [170].

There is recent evidence to suggest that LRP may act as a multifunctional receptor that transports diverse ligands. α_2 -macroglobulin is a tetrameric serum protein that binds proteases thereby removing them from the circulation. Once proteases are bound, α_2 -macroglobulin undergoes a conformational change and becomes activated. α_2 -macroglobulin protease complexes are removed from the circulation by a receptor mediated process. The receptor for activated α_2 -macroglobulin was purified and shown to be a single Ca^{2+} binding subunit of 380-420 kDa [171],[172],[173]. Protein sequencing of 10 peptide fragments of the α_2 -macroglobulin receptor aligned with the derived protein sequence from the LRP cDNA [174]. Two non-covalently associated proteins of 440 and 85 kDa were immunoprecipitated by a monoclonal antibody directed against the α_2 -macroglobulin receptor. These proteins correspond to the products of post-translational cleavage of intact LRP-600 at RHRR to generate LRP-515 and LRP-85 [167].

High affinity binding of activated α_2 -macroglobulin (1 nM) and rabbit β -VLDL enriched with recombinant apo E (0.2 nM) to rat LRP has recently been reported [175]. The addition of 20 $\mu\text{g}/\text{ml}$ activated α_2 -macroglobulin abolished specific binding but recombinant β -VLDL resulted in displacement of only 80% of specific ^{125}I -activated α_2 -macroglobulin binding. The authors suggested that these two ligands bound to independent sites of LRP and that steric hindrance resulted in the partial inhibition of ^{125}I -activated α_2 -macroglobulin binding. The effect of unlabelled activated α_2 -macroglobulin on ^{125}I -recombinant β -VLDL binding was not tested. However, the addition of increasing concentrations of activated α_2 -macroglobulin inhibited enhancement of cholesterol esterification caused by apo E-enriched β -VLDL in FH fibroblasts. The

degradation of iodinated, activated α_2 -macroglobulin was effectively competed by 200 $\mu\text{g/ml}$ unlabelled activated α_2 -macroglobulin, partially competed (60%) by the addition of recombinant apo E-enriched β -VLDL. The addition of β -VLDL or asialofetuin had no effect on the degradation of ^{125}I -activated α_2 -macroglobulin in FH fibroblasts.

The effect of activated α_2 -macroglobulin on chylomicron remnant uptake was tested in an *in vivo* experiment. Canine chylomicrons were injected into hepatectomized rabbits. Chylomicron remnants were isolated by ultracentrifugation which were in turn injected into mice for this study. Activated α_2 -macroglobulin caused 10% inhibition of chylomicron remnant clearance and 20% inhibition of chylomicron remnant hepatic uptake [175]. The addition of exogenous apo E to the chylomicron remnants enhanced clearance and uptake, however, these effects were still inhibited in the presence of activated α_2 -macroglobulin. These *in vivo* experiments are not conclusive, as only two mice were injected for each experiment, dramatic changes were not observed and the validity of testing canine chylomicron remnants prepared in rabbits in an *in vivo* clearance study in mice is in question.

I. E. 3. OTHER MEMBERS OF THE LDL RECEPTOR FAMILY

Heymann nephritis is a rat model of human membranous glomerulonephritis which relies on the interaction of a membrane glycoprotein (gp 330) with autoantibodies. A partial cDNA has been isolated for gp 330 which shows similarity to the A-type repeats and EGF precursor B-type repeats found on the LDL receptor [176]. In addition, gp 330 contains three repeats of the NPXY internalization sequence and is found localized in coated pits [177]. The role of gp 330 as a membrane receptor is a subject of debate.

LDL receptor homologs Notch, Delta and lin-12 have been isolated from *Drosophila* [178], [179] and *Caenorhabditis elegans* [180] respectively. These proteins may play a role in cell-cell interactions in these organisms.

I. F. RECEPTOR MEDIATED ENDOCYTOSIS

The role of receptor mediated endocytosis in intracellular transport was first described for the uptake of human LDL by cultured human fibroblasts [181], [182]. The evidence for a receptor mediated endocytic pathway regulating oocyte growth in oviparous species has emerged both from morphological and biochemical studies.

I. F. 1. RECEPTOR MEDIATED UPTAKE OF VERY LOW DENSITY LIPOPROTEIN

Elegant morphological studies were completed by Gilbert and co-workers describing a coated pit, coated vesicle pathway for VLDL uptake in the domestic hen [183]. Oocyte follicles in the the rapid growth phase were examined by electron microscopy. VLDL particles were shown to traverse the basal lamina, migrate between granulosa cells and reach the oocyte membrane where they are concentrated in coated pits. Invagination of the membrane led to formation of coated vesicles. These vesicles eventually lose their clathrin coat and fuse to form yolk spheres [183]. *In vitro* experiments using membranous sheets showed that VLDL binding was heparin- and EDTA-sensitive [18]. Furthermore, immature hen LDL, lacking apo VLDL-II, bound to the membranous sheets indicating that the receptor-mediated binding of cholesterol-rich lipoproteins in avian species is likely mediated through apo B [18].

Immunodiffusion experiments suggested that VLDL isolated from egg yolk was related to both plasma VLDL and LDL, but not HDL [32]. An *in vivo* labelling experiment showed that ^3H -leucine could be recovered as a peak of radioactivity in an egg 4 days after injection into a hen [184]. ^3H -labelled VLDL was isolated and injected into another series of hens. A peak of radioactivity was localized exclusively in the VLDL fraction from eggs four days post-injection [184]. Identity of plasma and yolk apo VLDL-II indicates that this apolipoprotein is not processed upon oocyte uptake [185].

No direct evidence for a specific receptor existed until two studies by Roth and co-workers. In 1981, the binding of avian lipoproteins to oocyte membrane sheets was tested

[186]. High affinity binding of VLDL (K_d of 12.6 nM) and co-operative binding of LDL was observed. The pH optima of binding was shown to be 5.3 and 7.3 for VLDL and LDL, respectively. Unlabelled LDL was unable to compete for ^{125}I -VLDL binding, but unlabelled HDL showed partial competition. In contrast, unlabelled VLDL competed for ^{125}I -LDL binding; unlabelled HDL showed no competition. The data from these experiments led the investigators to conclude that apo VLDL-II confers VLDL binding. Further experiments using a rapid centrifugation assay and homogenized membrane preparations revealed a different K_d of 46-48 nM and showed partial dependency on Ca^{2+} and dependence on Mg^{2+} for binding [187].

At no time during these experiments was the receptor described further. The molecular weight of the receptor was unknown and no attempts of purification had been reported in the literature. It is also interesting to note that no other studies have been conducted in oviparous (egg laying) higher vertebrate species on the role of lipoprotein transport into the developing oocyte. Certainly cholesterol laden lipoproteins are synthesized [79], but their role in oocyte growth has not been described in piscine, amphibian or reptilian species.

I. F. 2. RECEPTOR MEDIATED UPTAKE OF VITELLOGENIN

Using a similar approach, Roth and co-workers illustrated that a specific receptor mediated vitellogenin uptake into avian oocytes. Phosvitin bound to membranes with a K_d of 3.3 μM [188]. The putative phosvitin receptor was solubilized from oocyte membranes using the non-ionic detergent Triton X-100 and was shown to be a monomeric 116 kDa protein by affinity chromatography with phosvitin-Sepharose and chromatography of phosvitin-receptor complexes on a Biogel A-1.5 column [189]. In a subsequent study, optimal binding of VTG was observed at pH 6.0 and the interaction of ^{125}I -phosvitin with a membrane receptor was inhibited by prior treatment with proteases or fucosidase, implying that carbohydrate may play a role in phosvitin recognition [189]. The kinetic parameters of VTG and phosvitin binding were examined. VTG binds to oocyte

membranes with a K_d of 2.5 μ M while phosvitin binds with a K_d of 2.4 μ M [63]. Competitive binding studies revealed that phosvitin is a competitive inhibitor of ^{125} I-VTG binding and vice versa [63].

Later studies performed in our laboratory by Stefano Stifani have placed many of these observations in question. VTG binds to a 95 kDa oocyte membrane protein with a K_d of 96 μ g/ml (200 nM) [64]. Phosvitin was unable to displace ^{125} I-VTG binding in ligand blotting experiments. Later studies revealed that lipovitellin competed for ^{125} I-VTG binding to receptors solubilized from *Gallus* or *Xenopus* oocytes [58].

The central role that vitellogenin plays in oocyte growth is evident from a growing number of studies showing vitellogenin uptake from different species. VTG receptors have been described from locust [190], [191], [192], cockroach [193], [194], cecropian moth [195], tobacco hornworm [196], *Drosophila* [197], lobster [198], salmon [199] and *Xenopus* [200].

I. F. 3. RECEPTOR MEDIATED UPTAKE OF OTHER YOLK PRECURSORS

The similarity between yolk components and their hepatically synthesized precursors suggested that a number of other molecules were transported in the circulation and concentrated in the oocyte.

Williams showed the similarity between serum transferrin, a fraction isolated from egg yolk and conalbumin (found in the albumen) [10]. Tryptic digestions of all three proteins yielded similar patterns and amino acid analysis of transferrin and conalbumin were identical. Conalbumin, which is synthesized in the oviduct and concentrated in the egg white, had a higher electrophoretic mobility which was attributed to glycosylation differences.

Serum IgG and yolk IgG displayed identical isoelectric focussing mobility, as did F_c fragments isolated from serum and yolk [13]. A putative IgG receptor was initially isolated from yolk sac membranes with a K_d of 2-3 μ M and optimal binding was observed

at pH values ranging from 6.0-6.3 [12]. Further studies illustrated an interesting developmental pattern of the IgG receptor. Day 8 embryos express a low affinity receptor (K_d of 340 nM) but day 18 embryos have an additional high affinity component (K_d of 30 nM) [14]. Intact chicken IgG or chicken IgG F_C fragments competed for specific binding, while chicken IgG F_{ab} fragments showed no competition. Conalbumin or phosvitin were unable to compete; however, it would be interesting if the authors had tested the serum precursors transferrin and vitellogenin. Bovine IgG and goat IgG only competed at 100-fold higher concentrations than chicken IgG.

Yolk riboflavin binding protein lacks the carboxy terminal 13 amino acids of the serum form, suggesting that co- or post-transport processing has occurred [8]. Perhaps this modification is important for transport of riboflavin into the oocyte.

I. G. SCOPE OF THE THESIS

In 1985, only fragmentary evidence existed for a functional oocyte lipoprotein receptor. Morphological experiments by Gilbert and co-workers provided strong evidence for a receptor-mediated pathway for VLDL transport into the oocyte. Immunological experiments suggested that plasma VLDL was transported into the yolk proper. In addition, Roth and co-workers demonstrated a putative VLDL receptor from binding experiments with crude oocyte membrane fragments. No attempt to solubilize the receptor had been made or to characterize it further. Having a vast array of reagents available from the study of the mammalian LDL receptor and using the powerful method of ligand blotting, the goals for this research project were as follows:

- i) to provide evidence for a specific receptor for VLDL uptake into the oocyte
- ii) purify the binding activity and perform a kinetic evaluation of ligand binding to the purified receptor. In addition, protein sequencing should give valuable sequence data for design of oligonucleotide probes.
- iii) determine the role that this protein plays in oocyte growth
- iv) evaluate the role of this receptor versus receptors for transport of other yolk components such as vitellogenin and vitamin binding proteins.

FIGURE 11

CROSS-SECTION OF A CHICKEN FOLLICLE

Follicle and enclosed oocyte are shown. Not all layers are shown to scale. Reprinted from [22] with permission.

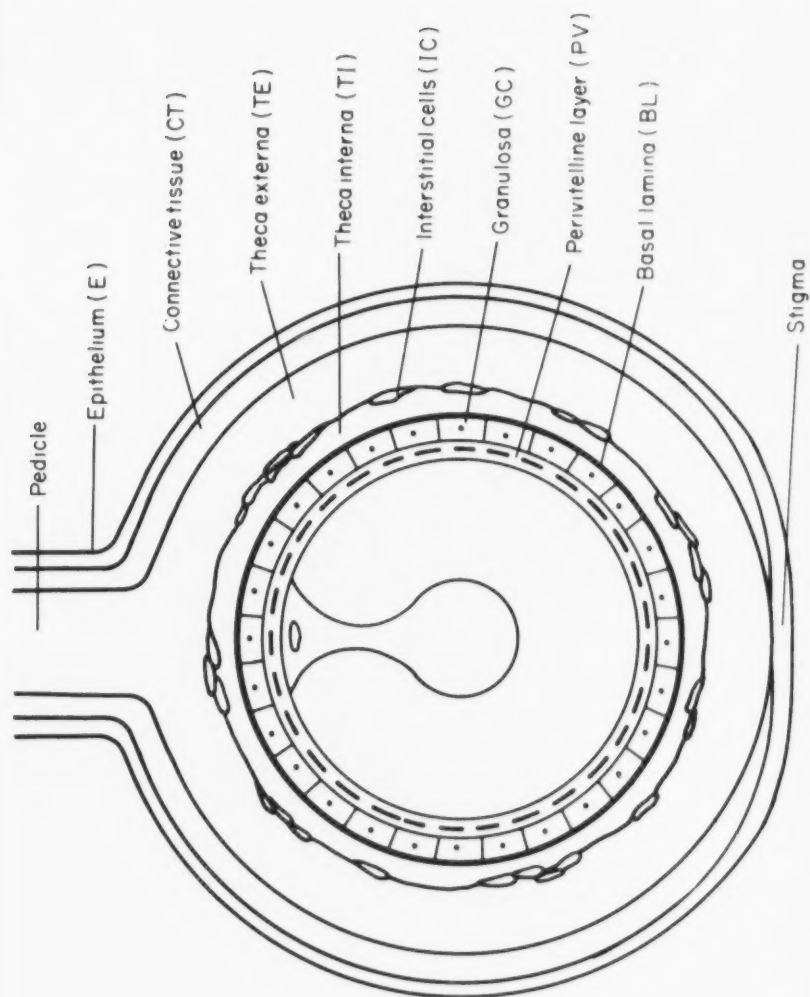


FIGURE I 2

ANATOMICAL RELATIONSHIPS OF THE REPRODUCTIVE ORGANS IN THE ABDOMINAL CAVITY OF THE DOMESTIC FOWL

1, follicle; 2, stigma; 3, post-ovulatory follicle; 4 , magnum; 5, muscular cord; 6, shell gland; 7, gut; 8, vagina; 9, ventral ligament; and 10, infundibulum. Reprinted from [22] with permission.

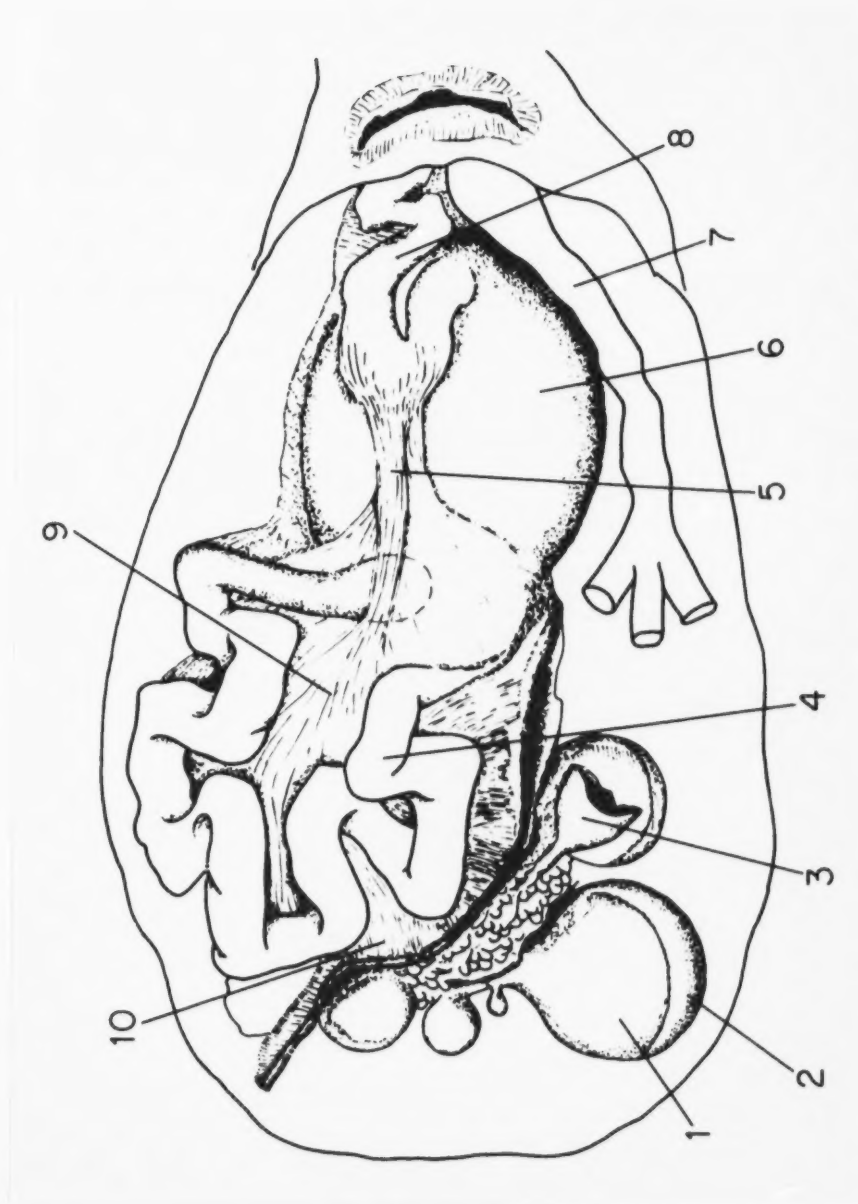


FIGURE 13

DOMAIN STRUCTURE OF THE LDL RECEPTOR

The five domains of the LDL receptor are shown. Repeats 2-7 are believed to constitute the major binding site for apo B-100 of LDL. This binding site is divided into two domains with repeats 2-4 and repeats 5-7 lying at the centre of a 2-fold axis of symmetry that is divided by an 8-amino acid linker sequence. Reprinted from [152] with permission.

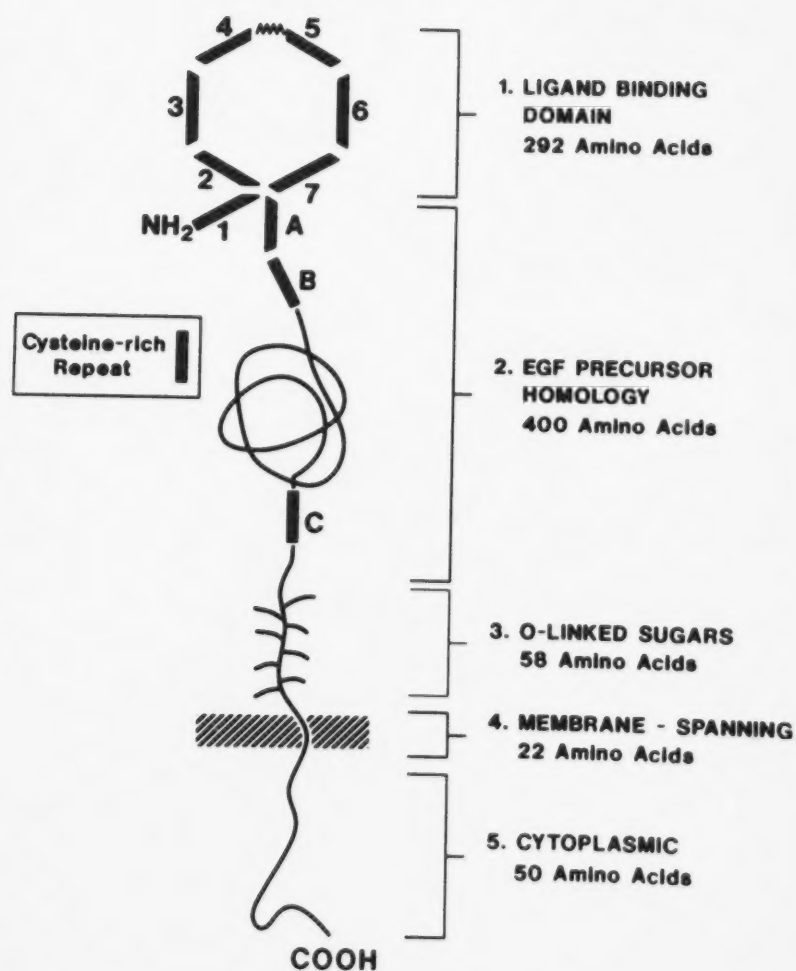
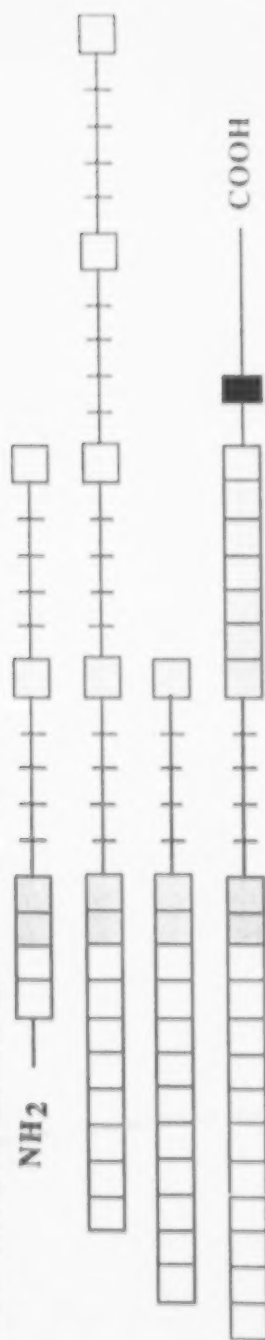


FIGURE 14

COMPARISON OF THE DOMAIN STRUCTURE OF THE LDL RECEPTOR FAMILY

Alignment of the LRP with the LDL receptor and the EGF precursor. The LRP has been broken into four pieces to emphasize the similarity with the other molecules. A-type repeats are shown in clear boxes, B-type repeats are shown in stippled boxes, the vertical lines illustrate YWTD sequences and the transmembrane domain is shown in black. Adapted from [166].

LDL Receptor-Related Protein (LRP)



LDL Receptor



EGF Precursor



CHAPTER II
MATERIALS AND METHODS

II.A. MATERIALS

We obtained PMSF, aprotinin, leupeptin, 17-ethinylestradiol, Freund's complete adjuvant, Freund's incomplete adjuvant, Triton X-100, Triton X-114, Nonidet P-40, octyl β -D-glucoside, IODO-GEN, bovine serum albumin, egg phosphatidyl choline, neuraminidase, biotinylated concanavalin A, biotinylated *Tetragonolobus purpureas*, biotinylated *Ulex europaeus* UEA-I, biotinylated *Bandeiraea simplicifolia* BS-1, biotinylated *Arachis hypogaea*, biotinylated *Triticum vulgare*, peroxidase-conjugated *Bandeiraea simplicifolia* BS-1, peroxidase-conjugated concanavalin A, peroxidase-conjugated soyabean lectin, peroxidase-conjugated wheat germ lectin and trypsin from Sigma; DEAE-Cellulose DE-52 from Whatman; Na^{125}I (100 mCi/ml) was received from Edmonton Radiopharmaceutical Centre, Amersham or ICN; molecular weight standards from Bethesda Research Laboratories or Sigma; 3-((3-cholamidopropyl)dimethylammonia)-1-propane sulfonate (CHAPS), endoglycosidase F, endoglycosidase H, endoproteinase arg-C, endoproteinase glu-C, endoproteinase lys-C, and pronase from Boehringer Mannheim; O-glycanase and N-glycanase from Genzyme; CNBr-Activated Sepharose 4B, Sephacryl S-300, Sephacryl S-1000, and Sephadex G-25 columns PD-10 from Pharmacia; TransBlot nitrocellulose paper from BioRad; Immobilon-P polyvinylidene difluoride membrane and Millex HA 0.45 μm filters from Millipore; cellulose acetate membrane filters (0.45 μm) from Advantec, Japan (CO 45) or Schleicher and Schuell (OE 67); and suramin from FBA Pharmaceuticals, New York.

II.B. ANIMALS AND DIETS

White leghorn hens were purchased from the Department of Animal Science, University of Alberta and maintained on a layer mash. Roosters were obtained from the same source and were fed a grower mash. All chickens received a light period of 12 hr.

TABLE II 1

BUFFER MIXTURES

A	0.15 M NaCl and 0.2 mM EDTA (pH 7.4)
B	20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2 mM EDTA, 1 mM PMSF, 5 μ M leupeptin and 2.5 μ g/ml aprotinin
C	5 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2
D	16 mM sodium citrate (pH 5.5), 1 mM PMSF, 5 μ M leupeptin and 2.5 μ g/ml aprotinin.
E	50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 and 30 mM octyl glucoside.
F	50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 and 1 mM PMSF
G	250 mM Tris-maleate (pH 6.0), 2 mM CaCl_2 , 1 mM PMSF, 5 μ M leupeptin and 2.5 μ g/ml aprotinin
H	50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.1% (w/v) sodium dodecyl sulfate
I	50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 and 0.2% (v/v) Triton X-100
J	50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 and 5% (w/v) bovine serum albumin (BSA)
K	50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 , 0.05% (w/v) Triton X-100 and 5% (w/v) BSA
L	50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 and 5% (w/v) non-fat dry milk.
M	30 mM sodium citrate (pH 5.5), 0.75% (w/v) SDS, 1 mM PMSF and 1 mM DTT
N	190 mM sodium phosphate (pH 8.6), 1.2% (v/v) Nonidet P-40 and 0.16% (w/v) SDS
O	50 mM Tris-HCl (pH 8.0), 2 mM CaCl_2 , 1 mM PMSF and 0.2% (v/v) Triton X-100
P	50 mM Tris-HCl (pH 8.0), 1 M NaCl, 2 mM CaCl_2 , 1 mM PMSF and 0.2% (v/v) Triton X-100
Q	50 mM Tris-HCl (pH 8.0), 2 mM CaCl_2 , 1 mM PMSF and 30 mM octyl glucoside
R	50 mM Tris-HCl (pH 8.0) and 2 mM CaCl_2
S	25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM CaCl_2 and 1 mg/ml BSA

Oocytes were also collected during slaughter by permission of Lilydale Poultry Sales, Edmonton, Alberta. Adult female New Zealand white rabbits were used for antibody production.

II. C. LIPOPROTEIN AND LIGAND ISOLATION

The VLDL fractions were isolated from laying hens, whereas LDL and HDL were fractionated from normal roosters.

Blood was collected from birds into a cocktail of protease inhibitors to yield 10 mM EDTA, 1 mM PMSF, 5 μ M leupeptin and 2.5 μ g/ml aprotinin (pH 7.4). Plasma was obtained after centrifugation at 10 000 $\times$ g for 15 minutes at 4 °C. For VLDL isolation, the plasma was centrifuged for 24 hours at 200 000 $\times$ g at 4 °C. The floating, yellow layer was resuspended in 0.15 M NaCl, 0.2 mM EDTA (pH 7.4) (buffer A) and was subjected to a second ultracentrifugation as above. Chicken VLDL was recovered from the top of the tube.

Yolk VLDL was isolated as follows [201]. Yolk was collected from a fresh egg, separated from the white, rinsed under cold water, and the yolk was extruded by incision and mixed with 5 volumes of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2 mM EDTA, 1 mM PMSF, 5 μ M leupeptin, 2.5 μ g/ml aprotinin (buffer B) and subjected to sequential ultracentrifugation steps at 200 000 $\times$ g as described above for plasma VLDL.

Chicken LDL was isolated via sequential ultracentrifugation at 200 000 $\times$ g for 24 hours at 4 °C at d 1.21 g/ml (top recovered), then d 1.06 g/ml (top recovered). LDL was then collected after equilibrium density centrifugation as described [202]. Gradients of KBr were prepared as follows, from bottom to top: 3 ml of d 1.15 g/ml solution; 3 ml of d 1.06 g/ml solution; 3 ml of d 1.02 g/ml solution and 3 ml of the LDL sample that had been dialyzed against buffer A, which was used to make the above solutions. The LDL fraction

was isolated approximately 6 cm from the top of the tube, after centrifugation at 39 000 rpm in a Beckman SWTi 40 rotor at 39000 rpm for 18 hours at 4 °C.

Chicken HDL was prepared by consecutive ultracentrifugation steps at 200 000 x g for 24 hr at 4 °C at d 1.006 g/ml (bottom recovered), d 1.21 g/ml (top recovered), d 1.15 g/ml (bottom recovered) and d 1.21 (top recovered).

Human VLDL and human LDL were isolated in a similar fashion as described above for the corresponding avian lipoproteins. All lipoproteins were dialyzed against buffer A before use, and stored at 4 °C. Lipoprotein concentrations were determined using a modified Lowry procedure [202].

Apo E3/3 (isolated from the plasma of a subject with two E3 alleles) was kindly provided by Dr. Karl H. Weisgraber, Gladstone Foundation Laboratories, San Francisco. Reconstituted apo E3/3 was prepared as described by Dr. Steyrer of this laboratory [203]. Briefly, solubilized apo E3/3 in 0.1 M NH_4HCO_3 was reconstituted with a triglyceride-phospholipid emulsion [204], [205] to give a ratio of phosphatidylcholine to apo E of 4:1 (w/w). The solution was gently mixed and incubated at 37 °C for 10 min. After cooling on ice for 5 min, the procedure was repeated once and the particles were used immediately in competition experiments.

Reductive methylation of lipoproteins and VTG were completed by the addition of 1 μl 37% aqueous formaldehyde at 0 and 30 minutes, followed by the addition of 1 mg sodium borohydride every six minutes for 1 hr in 0.1 M borate, pH 9.0 [134]. Samples were incubated at 4 °C with gentle rotation followed by exhaustive dialysis against buffer A (lipoproteins) or 5 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 2 mM CaCl_2 (buffer C), (VTG).

For VTG purification, blood was withdrawn from a laying hen into 16 mM sodium citrate (pH 5.5), 1 mM PMSF, 5 μM leupeptin, 2.5 $\mu\text{g/ml}$ aprotinin (buffer D) and purified as described [64]. Plasma was collected after centrifugation at 10 000 x g for 10 min at 4 °C. The plasma was spun at 300 000 x g for 3 hr at 4 °C. The lower infranant and

bottom fractions were collected and diluted 1:10 with 50 mM sodium citrate (pH 5.5), 2 mM CaCl_2 , 1 mM PMSF and applied to a DEAE-52 Cellulose column (14 x 2.5 cm) at a flow rate of 75 ml/hr. The column was washed with two bed volumes of 100 mM sodium citrate (pH 5.5), 2 mM CaCl_2 , 1 mM PMSF. A 200 ml linear gradient from 0-250 mM NaCl was then applied to the column. Fractions containing peak absorbance were analyzed via SDS-polyacrylamide gel electrophoresis and dialyzed immediately against 5 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 for 3 hr. After dialysis, 5 μM leupeptin and 2.5 $\mu\text{g/ml}$ aprotinin were added, and aliquots were stored at -70°C .

II. D. LABELLING OF REAGENTS

Lipoproteins were iodinated using the iodine monochloride method [206]. One to five mg of protein were incubated with 200 mM glycine, pH 10 and incubated with 1 mCi of carrier-free Na^{125}I (100 mCi/ml). The tube was then stoppered, and 2-10 μg of ICl were added by injection and the suspension was applied to a Pharmacia PD-10 column preequilibrated with buffer A. Fractions containing radioactivity were pooled and dialyzed against the same buffer.

Vitellogenin, goat anti-rabbit IgG and protein A were labelled using IODO-GEN (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril) [207]. IODO-GEN (200 μg), dissolved in acetone, was allowed to evaporate in a vial. Protein was added, typically 1-2 mg and incubated with shaking at 23°C for 20 minutes. Labelled fractions were collected after chromatography and dialyzed as above against buffer C.

For reverse ligand blotting experiments, 95 kDa receptor was labelled using IODO-GEN as follows. Purified VLDL/VTG receptor (100-500 μg) was diluted to 1 ml in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 , 30 mM octyl glucoside (buffer E). The sample was applied to a Pharmacia PD-10 column preequilibrated with buffer E. Fractions containing radioactivity were pooled and aliquots were stored at -70°C .

II. E. PREPARATION OF MEMBRANE FRACTIONS

Oocyte follicles were collected during slaughter into a buffer containing 50 mM Tris HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 , 1 mM PMSF (buffer F). Oocytes (3-15 mm in diameter) were dissected from ovarian tissue and homogenized in buffer F plus 5 μM leupeptin, 2.5 $\mu\text{g/ml}$ aprotinin, using a Polytron for 30 sec at setting 5, followed by two 15 sec periods at setting 8. Large debris were removed by centrifugation at $10\,000 \times g$ for 10 min at 4°C and the resulting supernatant was subjected to centrifugation at $100\,000 \times g$ for 1 hr at 4°C . The membrane pellets were resuspended in buffer F plus protease inhibitors by aspiration through a 22-gauge needle and resedimented by centrifugation at $100\,000 \times g$ for 1 hr at 4°C ; this was repeated once to remove residually bound yolk proteins. The washed membrane pellet was quickly frozen in liquid N_2 and stored at -70°C for up to 6 months before use.

For larger oocytes, theca externa and theca interna were removed via dissection, and the yolk was extruded via an incision. The resulting membrane ghosts consisting of basement membrane, granulosa cells, perivitelline layer and oocyte membrane was rinsed in buffer F until free of yolk, minced with scissors and homogenized in buffer F plus leupeptin and aprotinin as described above. All other manipulations were performed as above to obtain membrane pellets.

For liver membrane preparations, livers were perfused with ice-cold buffer F and dissected free from the gall bladder. The tissue was minced using scissors and homogenized as above. Centrifugation was performed as described to give membrane pellets.

II. F. SOLUBILIZATION OF MEMBRANE PROTEINS

Membrane pellets (15-20 g) were suspended in 4.5 ml buffer containing 250 mM Tris-maleate (pH 6.0), 2 mM CaCl_2 , 1 mM PMSF, 5 μM leupeptin, 2.5 $\mu\text{g/ml}$ aprotinin (buffer G) by aspiration through a 22-gauge needle. A final volume of 9 ml was reached by the addition of water and an appropriate detergent to yield 5-10 mg of

protein/ml, 125 mM Tris-maleate (pH 6.0), 1 mM CaCl_2 , 0.5 mM PMSF, 2.5 μM leupeptin, 1.25 $\mu\text{g/ml}$ aprotinin. Various detergents were utilized to solubilize membrane proteins including: 36 mM octyl glucoside, 30 mM CHAPS, 1% (v/v) Nonidet P-40, 1% (v/v) Triton X-100 or 1% (v/v) Triton X-114 (final concentrations in the solubilization mixture). The suspension was incubated at 4 °C for 10 minutes prior to centrifugation at 100 000 $\times g$ for 1 hr. The supernatant was either quick frozen in liquid N_2 and stored at -70 °C or used directly in purification experiments.

For Triton X-114 phase separations, 750 μl of Triton X-114 solubilized oocyte or liver membrane extract was incubated in 750 μl of buffer containing 50 mM Tris-maleate (pH 6.0), 150 mM NaCl, 2 mM CaCl_2 , 0.06% (w/v) Triton X-114, 6% (w/v) sucrose for 10 min at 37 °C, followed by centrifugation at 10 000 $\times g$ for 5 min [208], [209]. The aqueous (supernatant) phase was removed, 200 μl 5% (w/v) Triton X-114 was added to give ~0.7% (w/v) final concentration of detergent, then the incubation and centrifugation steps were repeated. The detergent phase from this washing step was not saved. The detergent phase (pellet) from the original centrifugation step was washed with 1.0 ml of the buffer above, incubated at 37 °C for 10 min and respun. The aqueous phase was discarded. Both the aqueous and detergent phases were washed once more before aliquots were analyzed via SDS-polyacrylamide gel electrophoresis and ligand blotting.

II. G. ANTIBODY PREPARATION

Polyclonal rabbit anti-LDL receptor antibodies were raised by injection of 20 μg of phosphatidylcholine-acetone precipitated purified bovine LDL receptor [138] dissolved in 500 μl 0.15 M NaCl and emulsified with 500 μl of Freund's complete adjuvant on day 0, followed by immunizations on days 14, 21 and 28 with 10 μg receptor protein each in Freund's incomplete adjuvant.

An eluate fraction from VLDL-Sepharose 4B chromatography was separated via 4.5-18% SDS-polyacrylamide gel electrophoresis, stained briefly and destained. A band migrating at 95 kDa was excised and chopped into small fragments and extruded through

an 18-gauge needle. The gel pieces were incubated for 18 hours at 23 °C in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% (w/v) SDS (buffer H) [210]. The supernatant was removed and dialyzed exhaustively against a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl₂, 0.2% (v/v) Triton X-100 (buffer I). The antigen was prepared for injection by mixing equal volumes of the dialyzed eluate and Freund's adjuvant. The injection timetable followed the same regimen as described above.

Another band enriched from VLDL-Sepharose 4B chromatography, migrating at 380 kDa was eluted in a similar fashion as described above and used for injections.

An antibody directed against chicken apo B was prepared by J. Nimpf [201]. Apo B was isolated by electroelution [211] from 4.5-18% SDS-polyacrylamide gel electrophoresis on which delipidated plasma VLDL had been applied. This electroeluate was mixed with equal volumes of Freund's adjuvant and injected into a rabbit.

Delipidated yolk VLDL was separated via 4.5-18% SDS-PAGE and the major fragments yolk B1 (178 kDa), yolk B2 (79 kDa), yolk B3 (63 kDa) and yolk B4 (56 kDa) were eluted as described above for the 95 kDa receptor. Rabbits were immunized on day 0, 14, 21 and 28 as described above.

Serum was obtained from all animals on day 35 and every subsequent two weeks. The IgG fraction was purified via Protein A-Sepharose chromatography. Serum was diluted 1:1 with 0.1 M sodium phosphate (pH 8.0) and applied to a 0.5 x 4 cm Protein A-Sepharose column and allowed to recycle twice over the column. The column was then washed extensively with 0.1 M sodium phosphate (pH 8.0). Bound IgG was eluted with 0.1 M glycine, 1 M acetic acid (pH 2.5) into a buffer containing 1 M Tris-HCl (pH 8.0). Fractions were collected and dialyzed against 10 mM sodium phosphate (pH 8.0), 50 mM NaCl. Pre-immune IgG was prepared before immunization from each animal.

II. H. ELECTRON MICROSCOPY

VLDL samples were negatively stained with 2% sodium phosphotungstate (pH 7) and photographed in a Philips EM420 operated at 100 kV. The low-dose unit was employed to minimize specimen damage.

Oocytes and granulosa cell sheets [212] were prepared for conventional electron microscopy by fixation in 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 hr at 23 °C. After rinsing in phosphate buffered saline (PBS), the tissue was post-fixed in 1% buffered osmium tetroxide for 1 hr at 23 °C. After a further buffer rinse, the tissue was dehydrated in a graded series of ethanol solutions and propylene oxide and embedded in Araldite. Thin sections were cut, mounted on copper grids stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope.

Tissue was prepared for ultrastructural immunocytochemistry by fixation in a mixture of 2% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at 23 °C. Samples were then prepared for LR gold embedding as follows: the blocks of tissue were dehydrated in ethanol using the following series, all steps being carried out at -20 °C: 50%, 45 min; 70%, 45 min; 1:1 90% ethanol and LR gold resin, 1 hr; LR gold resin, overnight; LR gold resin plus initiator, 1 hr; LR gold resin plus initiator, overnight. This was then polymerized in capsules under a UV lamp (360 nm) for 24 hr. Thin sections were cut and mounted on uncoated nickel grids.

For cytochemical staining, grids were floated on a drop of filtered 1% bovine serum albumin (BSA) for 20 min and then transferred without washing to a drop of primary antibody, diluted if necessary with filtered PBS, for 2 hr at 23 °C. Control grids were floated on non-immune serum or PBS. After washing with jets of filtered PBS, each grid was floated on three successive drops of PBS for 10 min each. Grids were then floated on drops of protein A-gold (15 nm gold particles; Janssen or E-Y Laboratories Inc.) diluted 1:10 with filtered PBS for 1 hr at room temperature. Washing was performed using jets of filtered PBS followed by floating on drops of PBS as before. Final washing was carried

out with jets of filtered distilled water and blotting with filter paper. Grids were stained with uranyl acetate and lead citrate.

II. I. ELECTROPHORESIS AND TRANSFER

One dimensional SDS-PAGE was performed using a mini-gel system or a regular gel apparatus [213]. Electrophoresis was conducted at 200 V at 23 °C for 60 min for mini gels (8 x 6 x 0.15 cm) or at 35 mA per gel at 10 °C for 6 hr for regular gels (16 x 12 x 0.15 cm). Samples containing 50 mM dithiothreitol (DTT) were heated at 90 °C for 2 minutes. Gels were calibrated with the following molecular weight standards as indicated in the figures: myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β -lactoglobulin (18 kDa) and lysozyme (14 kDa) (BRL); myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa) (SIGMA). Gels were stained in 0.1% (w/v) Coomassie Blue, 5% (v/v) acetic acid, 25% (v/v) methanol for 2 hr and destained in 5% (v/v) acetic acid, 10% (v/v) methanol.

Gels were electrophoretically transferred to nitrocellulose or polyvinylidene difluoride (PVDF) in a buffer containing 20 mM Tris base, 150 mM glycine, 20% (v/v) methanol [214]. Large gels were transferred for 18 hr whereas mini gels were transferred for a 2 hr period in a BioRad mini-blot apparatus. Methanol was omitted for transfer of proteins over 150 kDa. Proteins were visualized on nitrocellulose by staining with Ponceau S in 3% (w/v) trichloroacetic acid briefly and destaining in 5% (v/v) acetic acid, 10% (v/v) methanol. Conventional Coomassie staining was useful for PVDF followed by destaining in 10% (v/v) acetic acid, 50% (v/v) methanol.

II. J. LIGAND AND IMMUNOBLOTTING

Ligand blotting was completed using a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 , 5% (w/v) BSA (buffer J) with additions of either ^{125}I -labelled VLDL or ^{125}I -VTG for 2 hr at 23 °C [151]. The incubation buffer for

reverse ligand blotting experiments contained 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 , 0.05% (v/v) Triton X-100, 5% (w/v) BSA (buffer K) [58]. Western blotting experiments utilized a buffer mixture containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl_2 , 5% (w/v) non-fat dry milk (buffer L). $^{45}\text{Ca}^{2+}$ -overlays were performed as described using 1 $\mu\text{Ci/ml}$ $^{45}\text{CaCl}_2$ in a buffer containing 10 mM imidazole-HCl (pH 6.8), 60 mM KCl, 5 mM MgCl_2 [215]. The concentrations and specific radioactivities of the ligands and antibodies used in the incubation mixtures are indicated in the figure legends. Autoradiographs were obtained by exposing the dried nitrocellulose paper to Kodak AR film at -70°C for the indicated times.

II. K. ENDOGLYCOSIDASE DIGESTIONS

Ten μg of VLDL-Sepharose 4B eluate was incubated in a buffer containing 30 mM sodium citrate (pH 5.5), 0.75% (w/v) SDS, 1 mM PMSF, 1 mM DTT (Buffer M) $\pm$ 0.2 U endoglycosidase (endo) H or endo F [144]. The samples were incubated at 37°C overnight. Alternatively, for N-glycanase digestions, 10 μg of VLDL-Sepharose eluate was incubated in 190 mM sodium phosphate (pH 8.6), 1.2% (v/v) Nonidet P-40, 0.16% (w/v) SDS (buffer N) $\pm$ 300 U N-glycanase for 18 hours at 37°C .

Ten μg of VLDL-Sepharose 4B eluate was incubated in 0.1 M sodium acetate (pH 6.0) $\pm$ 0.2 U neuraminidase for 18 hr at 37°C . The samples were then incubated in the presence or absence of 4 mU of O-glycanase for 24 hr at 37°C .

All samples were separated via 4.5-18% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with ^{125}I -VLDL.

II. L. LECTIN BLOTTING

Purified ligands, detergent solubilized oocyte membranes, purified 95 kDa receptor or purified oocyte LRP were resolved via 4.5-18% SDS-PAGE and transferred to nitrocellulose. These replicas were incubated in buffer J with 1 $\mu\text{g/ml}$ peroxidase-conjugated concanavalin A or 1 $\mu\text{g/ml}$ *Bandeiraea simplicifolia* BS-I followed by incubation with 4-chloro-1-naphthol to visualize the lectin interaction. Alternatively,

nitrocellulose strips were incubated with 1 µg/ml biotinylated concanavalin A, 1 µg/ml biotinylated *Bandeiraea simplicifolia*, 1 µg/ml biotinylated *Tetragonolobus purpureas*, 1 µg/ml biotinylated *Ulex europaeus* UEA-I, 1 µg/ml biotinylated *Arachis hypogaea* or 1 µg/ml biotinylated *Triticum vulgaris* in buffer J for 2 hr at 23 °C. This was followed by incubation with ¹²⁵I-labelled streptavidin (334 cpm/ng) for 2 hr at 23 °C. Autoradiographs were obtained after exposing dried nitrocellulose strips.

II. M. PROTEIN PURIFICATIONS

II. M. 1. VLDL-Sepharose 4B Chromatography

Plasma VLDL, prepared as described, was coupled to CNBr-activated Sepharose 4B according to manufacturer's instructions [216] by using 35 mg of plasma VLDL per g of dry gel. Alternatively, yolk VLDL was substituted for plasma VLDL. The VLDL-Sepharose 4B was stored at 4 °C in a buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM CaCl₂, 0.02% (w/v) NaN₃. Ten ml of Triton X-100 solubilized membranes were diluted to 40 ml with 50 mM Tris-HCl (pH 8.0), 2 mM CaCl₂, 1 mM PMSF, 0.2% (v/v) Triton X-100 (buffer O) and were applied to a column with dimensions of 1.5 x 10 cm containing the coupled matrix at a flow rate of 25 ml/hr at 4 °C. The sample was recycled over the column 5 times, prior to washing the column with 50 volumes of buffer O. Bound proteins were eluted using a gradient of 0-0.5 M NH₄OH. Fractions containing receptor protein were pooled and lyophilized. The lyophilizate was redissolved in buffer O for the following chromatographic step.

II. M. 2. Anti-oocyte receptor Sepharose 4B Chromatography

A polyclonal anti-oocyte receptor antibody was prepared as described above and coupled to CNBr-activated Sepharose 4B [216] using a ratio of 20 mg of Protein A-purified IgG per g of dry gel. The chromatography was performed similar to above, except that the gel was washed extensively with a buffer containing 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 2 mM CaCl₂, 1 mM PMSF, 0.2% (v/v) Triton X-100 (buffer P) and then prior to elution with 50 mM Tris-HCl (pH 8.0), 2 mM CaCl₂, 1 mM PMSF, 30 mM octyl

glucoside (buffer Q). The bound protein was eluted in the presence of 0.5 M NH_4OH . Appropriate fractions were pooled and lyophilized. Protein was dissolved in buffer Q and various fractions were analyzed via SDS-PAGE and ligand blotting.

II. M. 3. Anti-oocyte LRP Sepharose 4B Chromatography

A monospecific anti-oocyte LRP antibody was obtained by injection of eluted protein. Protein A-purified IgG was coupled to CNBr-activated Sepharose 4B as described above for anti-oocyte receptor IgG. Ten ml of Triton X-100 solubilized oocyte membranes were diluted to 40 ml with buffer O and applied to 1.5 x 10 cm column at a flow rate of 25 ml/hr at 4 °C. The sample was allowed to recycle over the column for at least 5 column volumes, and the column was washed exhaustively with buffer P. Before elution the column was rinsed free of salt using buffer Q and elution was performed in the presence of 0.5 M NH_4OH . The eluate was lyophilized, dissolved in buffer Q and analyzed via SDS-polyacrylamide gel electrophoresis.

Once anti-oocyte LRP Sepharose 4B chromatography was successful, the flow through was then applied to Anti-oocyte receptor Sepharose 4B directly, and VLDL-Sepharose 4B chromatography was no longer utilized for receptor purifications.

II. N. PROTEIN SEQUENCING

The oocyte receptor or oocyte LRP was purified as described, subjected to electrophoresis on a 4.5-18% SDS-polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose filter was stained with Ponceau S, the region where each protein bound was sliced out and the bound protein was digested on the membrane with trypsin [217]. The resulting peptides were released into the supernatant and separated by reverse-phase HPLC [218] using a Vydac C4 column (150 x 2.1 mm) in a Waters peptide analyzer. Prominent peptides were sequenced on an Applied Biosystems model 477A sequenator using standard operating procedures.

II. O. SOLID PHASE BINDING ASSAY

Octyl glucoside solubilized crude membranes in buffer G, immunoaffinity purified oocyte 95 kDa receptor in buffer Q or immunoaffinity purified oocyte LRP in buffer Q was mixed with 4 M NaCl, 1 mg/ml egg phosphatidylcholine (PC) liposomes to give a final concentration of 50-200 μ g/ml protein, 12.5 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 0.5 mM CaCl_2 , 0.5 mg/ml PC, 7.5 mM octyl glucoside [219]. To this suspension, 0.6 volumes of ice-cold acetone was added while vortexing. This sample was spun at 20 000 $\times g$ for 15 min at 4 °C. The pellet was resuspended with a 22-gauge needle only in 1.0-1.5 ml of 50 mM Tris-HCl (pH 8.0), 2 mM CaCl_2 (buffer R) and used immediately in the assay.

The standard assay mixture contained in a volume of 100 μ l: 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, 0.8 mM CaCl_2 , 16 mg/ml BSA, the indicated concentrations of ^{125}I -labelled ligand and the indicated fixed concentration of precipitated protein. Incubation was for 2 hr at 23 °C, and free ligand was separated from receptor-bound as follows. Cellulose acetate membranes (0.45 μ M) were soaked in 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM CaCl_2 , 1 mg/ml BSA (buffer S). The filter was washed with 3 ml of buffer S. Eighty μ l of sample was added to 3 ml buffer, vacuum was applied. The filter was then washed 3 times with 3 ml of buffer S each time. Vacuum was broken each time after the wash had been drawn through the filter. Filters were counted by a Beckman Gamma 5500 gamma counter.

II. P. PROTEASE DIGESTIONS

Affinity purified oocyte 95 kDa receptor or affinity purified oocyte LRP was incubated with endoproteinase arg-C (1:100), endoproteinase lys-C (1:100) endoproteinase glu-C (1:100), pronase (1:200), proteinase K (1:200), or trypsin (1:1000) for various times at 37 °C. Aliquots were resolved via SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed via ligand blotting with ^{125}I -VLDL or ^{125}I -VTG.

II. Q. OTHER METHODS

Protein concentrations were determined by the Lowry method [220]. The protein content of lipoproteins or samples containing detergent was calculated by a modification of the Lowry procedure [221] involving the addition of sodium deoxycholate and precipitation using trichloroacetic acid.

CHAPTER III
ISOLATION AND CHARACTERIZATION OF THE
OOCYTE 95 kDa VLDL RECEPTOR¹

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III.A. INTRODUCTION

The dramatic growth of the avian oocyte was first correlated to an induction of VLDL synthesis in the laying hen in 1954 [23]. Subsequently, it was discovered that the level of a major plasma phosphoprotein, later identified as VTG, was raised markedly in serum from estrogen-treated roosters [69]. The synthesis of a number of serum proteins including VLDL, vitellogenin [42], retinol binding protein and riboflavin binding protein [74] were shown to be under estrogen regulation. Labelling studies had demonstrated that injected ^3H -VLDL was transported into the oocyte [184]. Immunological cross-reactivity was observed in VLDL isolated from the egg yolk and serum [32] and sequence identity between apo VLDL-II isolated from plasma and egg yolk has been described [185] illustrating that serum components must be transported into the oocyte, given the limited biosynthetic capacity of the primary oocyte.

Oocytes isolated during the rapid growth phase demonstrate incredible rates of protein uptake of 1.5-2 g of protein/day [6]. Morphological investigations have shown that the oocyte cytoplasmic membrane is lined with microvilli that contain coated pits that are actively involved in sequestering lipoprotein-like particles [183]. Subsequent invagination of the membrane gives rise to coated vesicles that migrate into the yolk periphery, lose their clathrin coat and fuse to form yolk granules. Therefore, the storage of triglyceride- and cholesterol-rich VLDL in the avian oocyte implies the existence of a receptor mediated pathway.

This chapter describes isolation of a 95 kDa oocyte VLDL receptor from solid phase binding assays as well as ligand blotting. Functional properties of this newly described protein are compared to the somatic mammalian LDL receptor. Analysis of the glycoprotein structure of the 95 kDa VLDL receptor is also provided.

III.B. RESULTS

III.B.1. ISOLATION AND CHARACTERIZATION OF CHICKEN LIPOPROTEINS

Lipoprotein fractions were isolated, purified and characterized from both sexes of *Gallus domesticus* at different stages of development. Figure III 1 shows delipidated apolipoproteins resolved via SDS-polyacrylamide gel electrophoresis from purified lipoproteins. Apolipoprotein AI (*lane A*), a 28 kDa protein is found in untreated rooster HDL [80]. Several centrifugation steps were necessary in order to yield a HDL population free of contaminating lipoproteins, as has been previously observed [222]. LDL (*lane C*) isolated from laying hen contains apolipoprotein B only, whereas laying hen VLDL (*lane D*) contains apo VLDL-II in addition to apo B. Chicken apo B migrates above the myosin standard, and similar to mammalian apo B has a M_r in the vicinity of 500 kDa. Apo VLDL-II is a 9.5 kDa disulfide-linked homodimer [34], which under these experimental conditions is observed migrating in both dimeric and monomeric forms. This apolipoprotein can be isolated from nascent VLDL particles by detergent extraction as shown in *lane E*. Lipoprotein profiles and apolipoprotein distribution were identical from laying hens and estrogen-treated roosters (data not shown).

A VLDL preparation was examined by electron microscopy using negative staining with phosphotungstate. As shown in Figure III 2, a uniform population of spherical particles is observed with a mean diameter of 38 ± 0.8 nm [201].

III.B.2 BINDING OF ^{125}I -LABELLED CHICKEN VLDL TO OOCYTE MEMBRANE DETERGENT EXTRACTS

A solid phase filtration binding assay developed for analysis of human ^{125}I -LDL binding to cultured human fibroblasts [221] was adapted to analyze the interaction of chicken apo B-containing lipoproteins with putative binding sites from detergent solubilized oocyte membranes. Octyl glucoside was used to solubilize oocyte membranes that had previously been washed extensively with aqueous buffers to eliminate adhering yolk

material. The membrane extract was then diluted below the critical micelle concentration (CMC) using phospholipid-acetone precipitation [221], resuspended in aqueous buffer and used in the assay. Increasing concentrations of ^{125}I -labelled lipoprotein were incubated in the presence or absence of a 100-fold excess of unlabelled lipoprotein. After incubation for 2 hr at 23°C , receptor-bound ligand was separated from free ligand by filtration using $0.45\text{ }\mu\text{m}$ cellulose acetate filters.

Figure III 3A illustrates the interaction of laying hen ^{125}I -VLDL with detergent solubilized oocyte membranes. Saturable, high affinity binding was observed; in addition, the binding was shown to be specific as an excess of unlabelled VLDL competed for binding. The binding reaction was shown to be Ca^{2+} -dependent as 20 mM EDTA abolished ^{125}I -VLDL binding. Scatchard analysis [223] (Figure III 3B) revealed a K_d of $19\text{ }\mu\text{g/ml}$ and a B_{max} of $12.8\text{ }\mu\text{g/mg}$ protein. Assuming a M_r of 950 kDa for apo B and apo VLDL-II, a single class of binding sites is observed with a K_d of 20 nM.

III.B.3 CHARACTERIZATION OF THE 95 kDa OOCYTE RECEPTOR

III.B.3.A. Ligand blotting

Pronase sensitivity of ^{125}I -VLDL binding suggested that the specific binding observed in Figure III 3A could be attributed to a proteinaceous component. Ligand blotting [151] was utilized to identify a putative oocyte lipoprotein receptor. Detergent solubilized membrane extract was resolved via SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and the nitrocellulose sheets were incubated in the presence of estrogen-treated rooster ^{125}I -VLDL and various additions as noted in Figure III 4. As shown in *lane A*, ^{125}I -labelled chicken VLDL binds to a 95 kDa membrane protein. This interaction is competed with either a 100-fold excess of unlabelled chicken VLDL or unlabelled chicken LDL as illustrated in *lanes B* and *C*, respectively. Chicken HDL, containing apolipoprotein AI, does not compete for ^{125}I -VLDL binding, as observed in *lane D*. At a concentration of 5 mg/ml, suramin, a highly negatively charged organosulfate compound, blocks the binding of ^{125}I -VLDL to the 95 kDa receptor protein (*lane E*). The

autoradiograph in Figure III 4 was purposefully overexposed in order to visualize the bands in *lanes B* and *C*. Prior reduction with 50 mM DTT of the octyl glucoside solubilized oocyte membrane extract destroyed ^{125}I -VLDL binding activity (data not shown).

This experiment would suggest that apo B-containing lipoproteins mediate binding to a 95 kDa oocyte receptor. In order to test this further, the binding of avian and mammalian lipoproteins was examined. Paired lanes containing purified bovine LDL receptor [138] and octyl glucoside solubilized oocyte membranes were separated via SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. These replicas were then incubated in the presence of individual labelled lipoprotein fractions as shown in Figure III 5. The bovine LDL receptor, which migrates at 130 kDa bound all lipoproteins [151]. Both avian VLDL and avian LDL bind strongly to the 95 kDa receptor (*lanes 2* and *4*). However, mammalian lipoproteins display weak interaction with the 95 kDa oocyte receptor. This was further verified by a solid phase filtration assay, in which Dr. George demonstrated that human ^{125}I -LDL bound to precipitated solubilized oocyte membrane proteins with a K_d of 84 nM [224]. A diffuse band was observed at 380 kDa from detergent solubilized oocyte membranes in this experiment (*lanes 2* and *6*). This interaction is described in later research (see Chapter VI).

III. B. 3. B. Immunoblotting

A polyclonal antibody was developed against phospholipid-acetone precipitated purified bovine LDL receptor [138] to test immunological similarity between lipoprotein receptors. As exhibited in Figure III 6, this antibody not only recognizes its native antigen, but also cross-reacts with the avian 95 kDa VLDL receptor. Incubations with pre-immune IgG failed to show any reactivity.

III. B. 3. C. Glycoprotein Nature

Ligand blotting experiments revealed that ^{125}I -VLDL bound to a 95 kDa receptor. However, the 95 kDa band observed is rather broad and diffuse, suggesting that the

receptor may be a glycoprotein (see Figure III 4). Incubations were conducted with various endoglycosidases to test for the presence of N- and O-linked carbohydrate followed by ligand blotting with ^{125}I -VLDL. The 95 kDa VLDL receptor has N-linked carbohydrate of a complex nature, demonstrated by its sensitivity to treatment with either endoglycosidase (endo) F (*lane C*) or N-glycanase (*lane F*), as well as its resistance to endo H (*lane B*). In order to test for O-linked carbohydrate, sialic acid residues must be previously removed with neuraminidase in order that O-glycanase may function. Neuraminidase treatment results in a slightly faster migrating form of the receptor (*lane I*), whereas sequential treatment with neuraminidase and O-glycanase (*lane J*) resulted in a receptor species demonstrating the greatest mobility of all. The addition of O-glycanase alone did not affect the migration of the receptor as shown in *lane K*. Other lower migrating proteins could be observed in the ligand blot of Figure III 7. These were not further investigated, but probably represent receptor aggregates [225] or related receptor species [166].

III. B. 3. D. Lectin Blotting

In order to further characterize the carbohydrate structure of the 95 kDa oocyte VLDL receptor, lectin blotting experiments were completed as shown in Figure III 8. A number of different peroxidase-conjugated lectins were incubated with nitrocellulose strips containing either octyl glucoside solubilized oocyte membranes or partially purified 95 kDa receptor. Concanavalin A recognizes α -D-mannose and α -D-glucose residues and as observed in *lane A* binds to many proteins in detergent solubilized oocyte membranes. *Bandeiraea simplicifolia* BS-I recognizes three major bands in oocyte membrane extract (*lane B*), but only one major band migrating at 95 kDa in a partially purified receptor preparation (*lane C*). This lectin binds to proteins containing α -D-galactose or β -D-galactose residues. The identity of the single band in *lane C* with the 95 kDa receptor was confirmed by ligand blotting with ^{125}I -VLDL to a nitrocellulose strip from the same gel (*lane D*). Incubations with soyabean lectin which recognizes D-galNAc showed only one

band migrating at > 300 kDa whereas wheat germ lectin which binds to proteins containing (D-glcNAc)₂ or NeuNAc residues recognized many bands in crude extract including the 95 kDa receptor (data not shown).

Incubations with peroxidase-labelled *Bandeiraea simplifolia* BS-I in the presence or absence of either 0.2 M raffinose or 0.2 M lactose were carried out to determine if this lectin recognized α - or β -linked galactose residues. Since raffinose, an α -linked trisaccharide, competes for *Bandeiraea simplifolia* BS-I binding (data not shown), it is evident that the 95 kDa receptor contained α -linked galactose residues.

III.C. DISCUSSION

Experiments presented within this chapter provide the first rigorous biochemical demonstration of an avian lipoprotein receptor. A 95 kDa integral membrane glycoprotein bound apo B-containing lipoproteins, VLDL and LDL. This protein was solubilized from membrane preparations prepared from oocyte follicles which consisted of a membranous array including the oocyte membrane, granulosa cells and the acellular perivitelline layer and basal lamina. Previously, morphological studies provided evidence that lipoprotein-like particles penetrated the basal lamina and were transported into the oocyte via coated structures [183]. This result was confirmed in an *in vitro* setting in which immature hen LDL and laying hen VLDL bound to isolated granulosa cell sheets [18]. Binding assays with radiolabelled lipoproteins and oocyte membrane fragments suggested that independent receptor binding sites existed for VLDL and LDL [186]. However, no studies have been reported to solubilize and further characterize the receptor mediating lipoprotein transport into the avian oocyte. Here, a 95 kDa receptor was isolated by detergent solubilization of avian oocyte membranes using the non-ionic detergent octyl glucoside. The binding interaction was characterized using ligand blotting, a solid phase binding assay, and immunoblotting.

The first step in studying a ligand-receptor system is to have a purified source of ligand. A dramatic rise in VLDL synthesis occurs during the onset of egg laying [23] or

upon estrogen treatment of roosters [24]. Ultracentrifugation has been used to separate lipoproteins based on their individual buoyant density. However, it has not been clear in previous studies that pure lipoprotein fractions were isolated [222], [186]. Therefore, consecutive ultracentrifugation steps must be employed in order to obtain discrete lipoprotein fractions. Individual delipidated fractions contained the appropriate apolipoproteins as previously described [79]. A step gradient [202] was the sole means of obtaining pure preparations of chicken LDL.

The receptor activity was first identified using a solid phase binding assay originally designed for the mammalian LDL receptor [219]. This assay relies on detergent solubilization using a non-ionic detergent with a high CMC. Subsequent dilution of the solution and precipitation with phosphatidylcholine and acetone gives a suspension that can be separated by centrifugation. The pellet is resuspended in aqueous buffer and then incubated with increasing concentrations of radiolabelled ligand in the presence or absence of unlabelled ligand. After a 2 hr incubation period, receptor-bound ligand is separated from free ligand by filtration, using a 0.45 μM cellulose acetate filter. High affinity, saturable, specific binding of ^{125}I -VLDL was observed. Scatchard analysis of this data revealed a K_D of 20 nM. Curvature of the Scatchard plot was noted. This was not characterized further at this time, but could represent multiple binding sites, negative cooperativity or other complex phenomena. Kinetic analysis from earlier studies revealed K_D values of 12.6 nM [186] and 45.8-47.8 nM [187]. The discrepancy in the values is not clear as the methodology used in both studies was similar. The specific binding activity of the receptor from crude oocyte membranes was quite low from Roth's studies, 1.4 $\mu\text{g}/\text{mg}$ [186] and 2 $\mu\text{g}/\text{mg}$ [187] suggesting that their membrane preparations were contaminated with endogenous yolk proteins. Significant enrichment of receptor binding was achieved through extensive washing of oocyte membranes with aqueous buffers, solubilization using octyl glucoside and elimination of soluble proteins after phospholipid-acetone precipitation. These manipulations led to a membrane preparation with a 6-fold higher

specific activity. As shown for the mammalian LDL receptor [138], the binding of VLDL is Ca^{2+} -dependent since 20 mM EDTA gives rise to non-specific binding.

In studies performed in our laboratory by Dr. George, essentially no difference was observed in the affinity of LDL or VLDL isolated from estrogen-treated roosters or laying hens for the 95 kDa receptor [224]. Optimal binding was shown to occur at pH 8.0 and kinetic values were similar at 4, 23 and 37 °C [224]. Heating of solubilized oocyte membrane proteins at 90 °C for 5 min destroyed binding activity as did incubation with 25 µg/ml Pronase [224]. A time course experiment revealed that binding of ^{125}I -VLDL to precipitated octyl glucoside extracts reached completion by 40 min and no measurable dissociation occurred over a 4 hr period [224].

The binding activity was abolished by treatment with Pronase, providing strong evidence that it was protein-mediated. Ligand blotting revealed that ^{125}I -VLDL bound to a 95 kDa membrane protein and this interaction could be competed with a 100-fold excess of unlabelled VLDL or LDL. This suggests that apo B mediates binding, as LDL is devoid of apo VLDL-II. Rooster HDL, which contains apo AI, is unable to compete for ^{125}I -VLDL binding. This contrasts with studies by Krumins and Roth [186], who showed that HDL competed for ^{125}I -VLDL binding, but not for ^{125}I -LDL binding. This led the authors to conclude that apo VLDL-II mediated VLDL binding whereas apo B was responsible for LDL binding to putative oocyte receptors. However, no analysis of lipoprotein fractions was presented in their study, suggesting contamination of the HDL fraction with apo B-containing lipoproteins. Repeated centrifugation of HDL as described in Chapter II was required to isolate a fraction containing only apo AI.

Gilbert and co-workers presented evidence that both immature hen LDL and laying hen VLDL bound to granulosa cell sheets [18]. Therefore, they speculated that apo B mediated binding of both ligands to oocyte membrane binding sites. This controversy was resolved by Dr. Nimpf in our laboratory who demonstrated that reconstituted apo VLDL-II was unable to displace either ^{125}I -VLDL or ^{125}I -LDL binding to receptor binding sites in

quantitative binding assays [202]. Suramin is a highly charged molecule that has been shown to block ligand-receptor interactions in the low density lipoprotein receptor [138], locust vitellogenin receptor [226], and platelet-derived growth factor receptor systems [227]. Receptor binding is inhibited by the addition of 5 mg/ml suramin to the incubation mixture.

The evolutionary conservation of lipoprotein receptors was tested by ligand blotting of avian and human lipoproteins to their respective receptors. The mammalian LDL receptor bound human LDL and VLDL as well as chicken VLDL and LDL. Human lipoproteins interacted weakly with the avian 95 kDa receptor, and this was verified by a quantitative binding assay [224]. This is the first demonstrated binding of avian lipoproteins to a mammalian LDL receptor and illustrates that a functional ligand binding domain of lipoprotein receptors has been conserved.

Lipoprotein receptors share many similar functional properties. Ligand binding to the 95 kDa oocyte VLDL receptor and the mammalian 130 kDa somatic receptor is Ca^{2+} -dependent, and sensitive to heating, reduction, suramin or Pronase treatment. Immunological similarity was also illustrated, as a polyclonal antibody raised against the purified bovine LDL receptor recognized the avian 95 kDa VLDL receptor. However, this antibody does not recognize the avian 130 kDa LDL receptor which is expressed on fibroblasts [228] and granulosa cells [229]. Therefore, the oocyte 95 kDa VLDL receptor is more closely related to the mammalian LDL receptor than its somatic counterpart.

The mammalian LDL receptor contains both N- and O-linked carbohydrate. One N-linked complex carbohydrate chain and 15 O-linked chains primarily are located primarily in one region of the the LDL receptor [145]. The 95 kDa oocyte receptor has asparagine N-linked carbohydrate of a complex structure demonstrated by resistance to endo H. O-linked carbohydrate is also present on the receptor, however, the O-deglycosylated receptor migrates at 90 kDa. This may indicate that the O-linked carbohydrate domain found on the mammalian LDL receptor, responsible for a 40 kDa

mobility shift, is not found in the oocyte receptor and only a few disperse O-linked chains are found on this protein. The 95 kDa receptor appears to express a unique α -linked galactose structure. Carbohydrate does not appear to play a functional role in ligand binding since various deglycosylated forms were still capable of ligand binding.

In summary, this chapter has illustrated that a specific oocyte derived receptor has evolved for transport of lipoprotein into the yolk. This protein shares many properties with the mammalian LDL receptor. This cell-specific protein functions in parallel with the 130 kDa receptor, a protein which is expressed in somatic tissues such as fibroblasts and granulosa cells and plays an integral role in maintenance of normal cholesterol homeostasis in these tissues.

FIGURE III 1

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF CHICKEN LIPOPROTEINS

Chicken lipoprotein fractions were purified as described in Chapter II. C and aliquots were delipidated by extraction with 20 volumes of ice-cold chloroform/methanol (2:1, v/v) prior to electrophoresis on a 5-20% SDS-polyacrylamide gradient gel. Samples contained 10 mM dithiothreitol and were heated to 90 ° C for 5 min. *Lane A*, 6 µg of protein of rooster HDL; *lane B*, M_r standards as indicated myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; β -lactoglobulin, 18 kDa; and lysozyme, 14 kDa. *lane C*, 10 µg of protein of laying hen LDL; *lane D*, 18 µg of protein of VLDL from estrogen-treated rooster; *lane E*, 8 µg of apolipoprotein VLDL-II. The proteins were stained with Coomassie Blue.



FIGURE III 2

ELECTRON MICROSCOPY OF LAYING HEN VLDL

Plasma VLDL was prepared as described in "Materials and Methods". VLDL samples were negatively stained with 2% sodium phosphotungstate and processed for electron microscopy. The instrumental magnification was x60000.

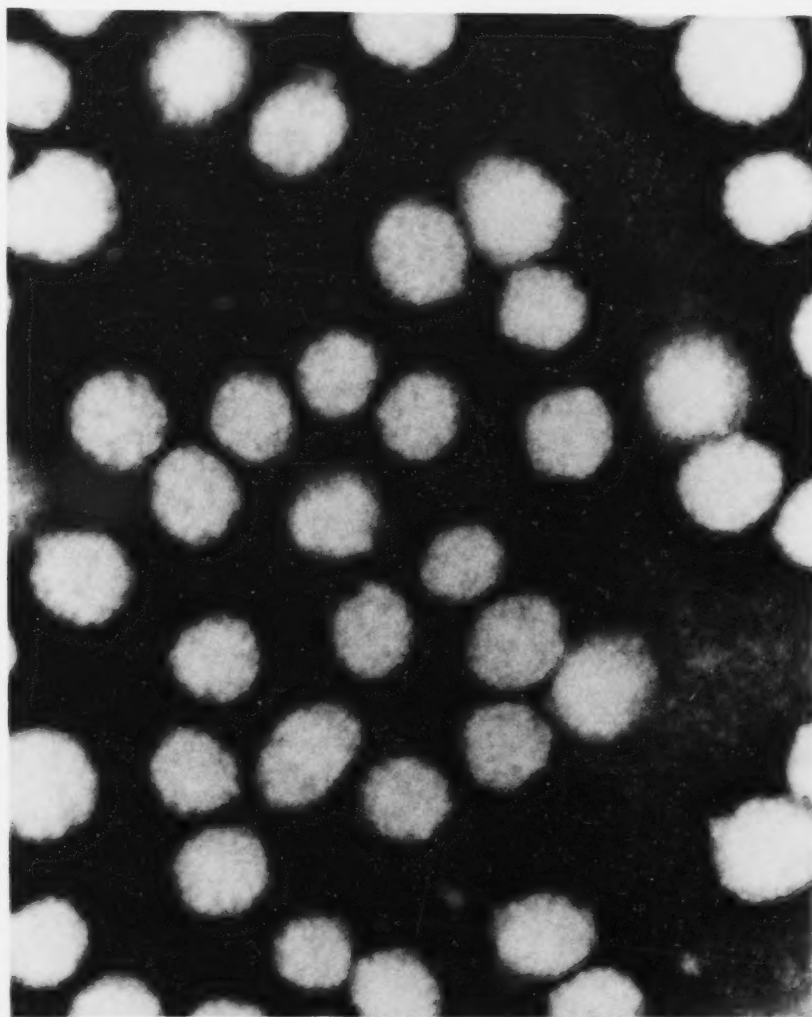


FIGURE III 3

SATURATION CURVE AND SCATCHARD PLOT FOR THE BINDING OF CHICKEN ^{125}I -VLDL TO CHICKEN OOCYTE MEMBRANE OCTYL GLUCOSIDE EXTRACTS

(A) Each assay tube contained the standard mixture (100 μl) with 12 μg of protein of precipitated octyl glucoside extract and the indicated concentration of radiolabelled ligand in the presence (■) or absence (●) of 7.1 mg/ml VLDL. Incubations were also carried out in the presence of 20 mM EDTA (○). The specific activity of the ^{125}I -VLDL was 51 cpm/ng. The amount of receptor-bound radiolabelled ligand was determined as described in Chapter II.O. High affinity binding (□) was calculated by subtracting non-specific binding (■) from total binding (●). Each data point represents the average of duplicate determinations.

(B) Binding data were analyzed by the method of Scatchard [223]. The ratio bound/free is the amount of bound ^{125}I -VLDL (μg of protein) divided by the amount of unbound protein in the reaction mixture (μg of protein).

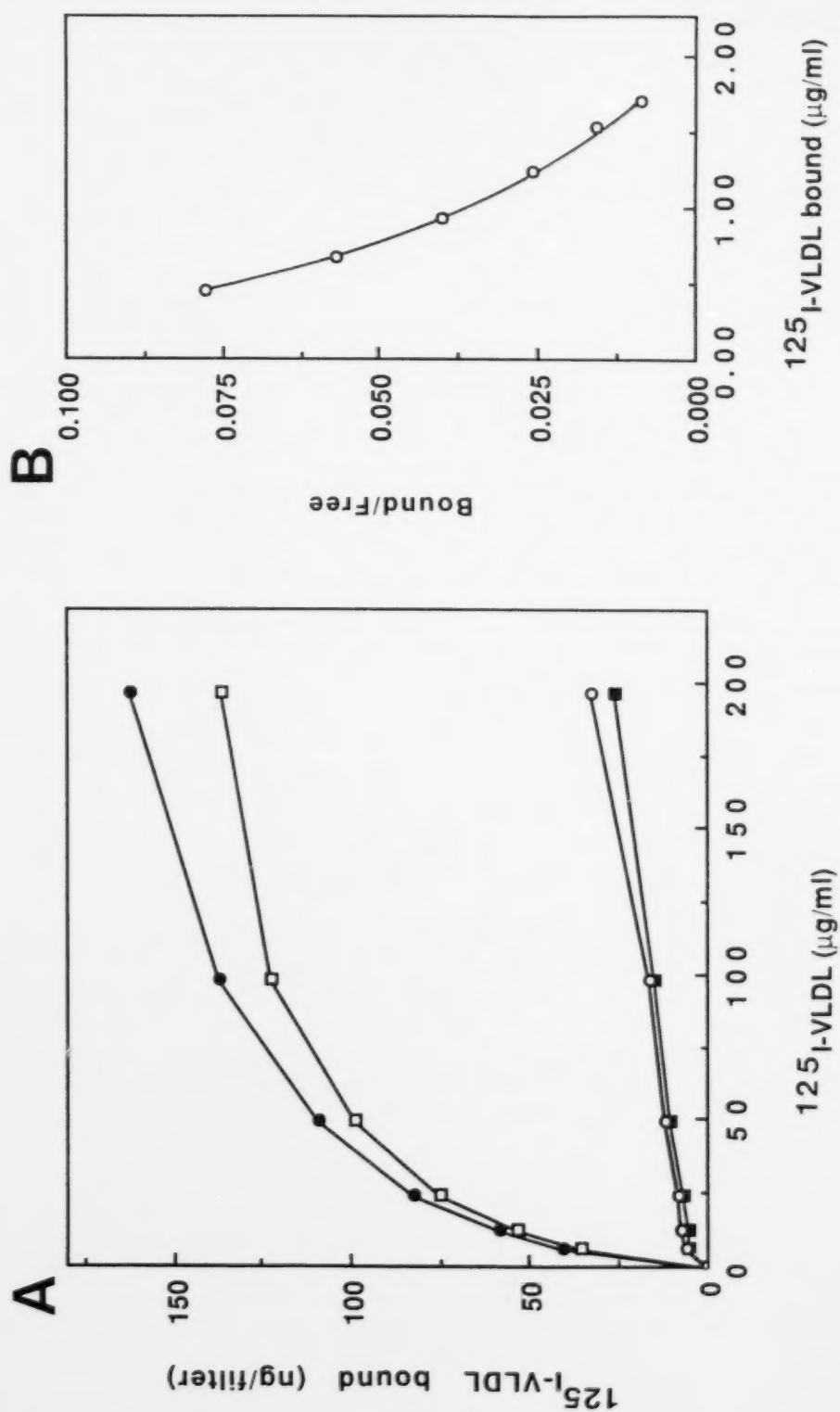


FIGURE III 4

LIGAND BLOTTING OF OOCYTE MEMBRANE OCTYL GLUCOSIDE EXTRACTS

Oocyte membrane octyl glucoside extract (17 μ g of protein/lane) was subjected to electrophoresis in a 4.5-18% SDS-polyacrylamide gradient gel under non-reducing conditions, followed by transfer to nitrocellulose. Ligand blotting was performed as described in Chapter II.J. The strips were incubated in buffer with 5 μ g/ml of ^{125} I-VLDL (180 cpm/ng) and the following additions: *A*, none; *B*, 0.5 mg/ml unlabelled chicken VLDL; *C*, 0.5 mg/ml unlabelled chicken LDL; *D*, 0.5 mg/ml unlabelled chicken HDL; and *E*, 5 mg/ml suramin. Exposure to XAR-5 film was for 24 hr at 23 °C. M_r standards are indicated. The radioactive bands were cut out from the nitrocellulose strips and their radioactivity was determined. The radioactivity in an equally sized piece representing the background was determined for each strip and subtracted. The resulting radioactivities were 15620, 970, 930, 14870 and 30 cpm for lanes *A-E*, respectively.

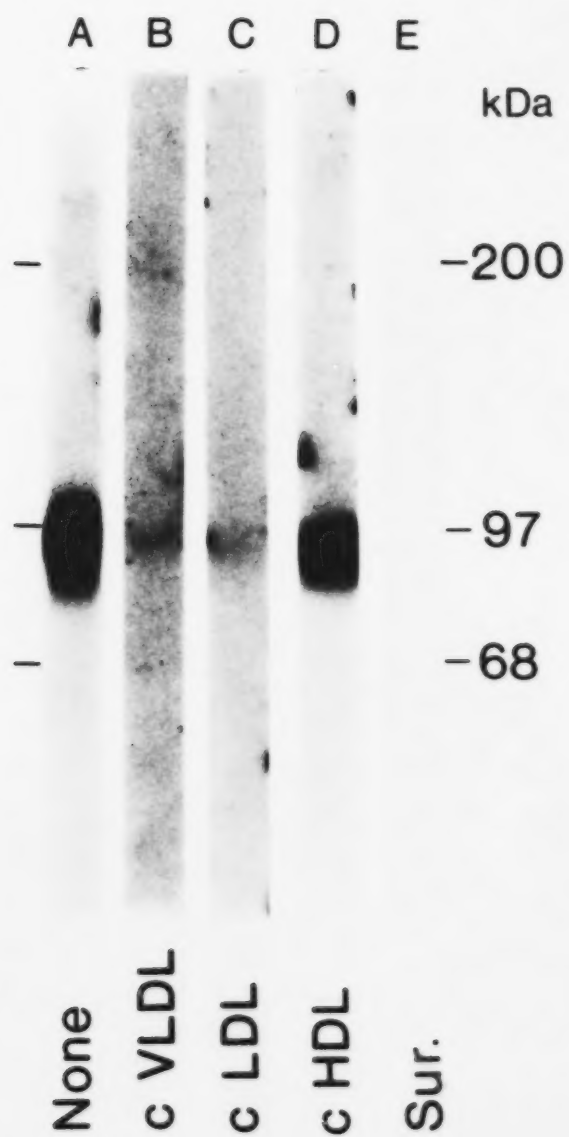


FIGURE III 5

LIGAND BLOTTING OF BOVINE AND CHICKEN LIPOPROTEIN RECEPTORS

Purified bovine receptor (0.8 $\mu\text{g}/\text{lane}$; *lanes 1, 3, 5, and 7*) and chicken oocyte membrane octyl glucoside extract (35 μg protein/*lane*; *lanes 2, 4, 6 and 8*) were subjected to electrophoresis on 4.5-18% SDS-polyacrylamide gradient gels under non-reducing conditions, followed by transfer to nitrocellulose, and ligand blotting as described in Chapter II.J. The strips were incubated in buffer containing the following radiolabelled lipoproteins: *lanes 1 and 2*, Chicken LDL (15 $\mu\text{g}/\text{ml}$; specific activity, 90 cpm/ng); *lanes 3 and 4*, chicken VLDL (10 $\mu\text{g}/\text{ml}$; specific activity, 115 cpm/ng); *lanes 5 and 6*, human VLDL (15 $\mu\text{g}/\text{ml}$; specific activity, 180 cpm/ng) and *lanes 7 and 8*, human LDL (15 $\mu\text{g}/\text{ml}$; specific activity, 250 cpm/ng). Exposure to XAR-5 film was for 18 hr at 23 ° C. M_r standards are indicated.

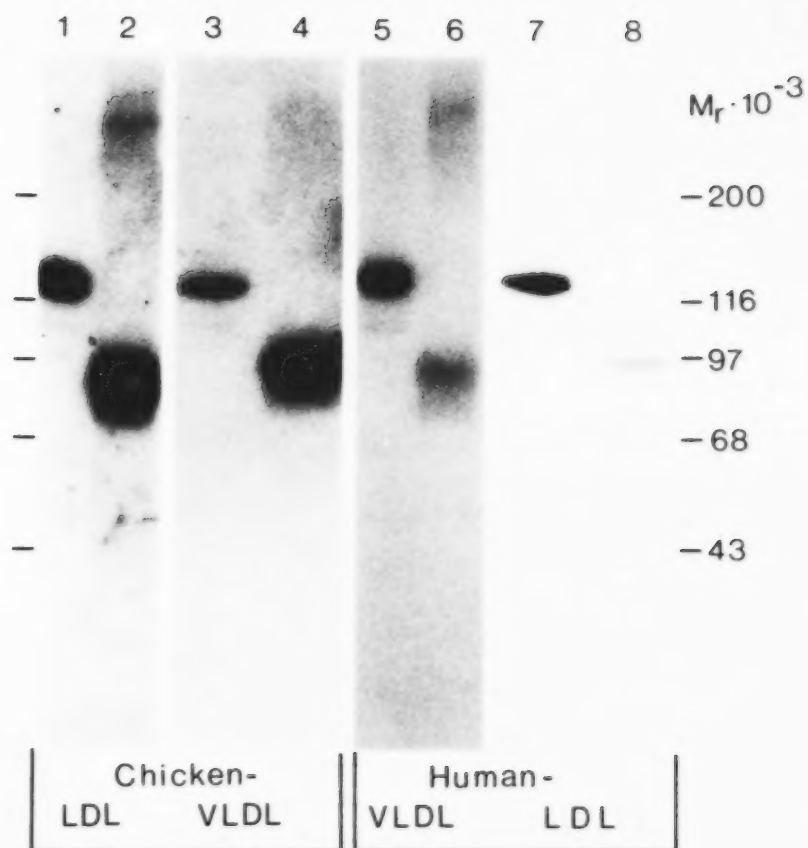


FIGURE III 6

IMMUNOBLOTTING OF LIPOPROTEIN RECEPTORS WITH ANTI-BOVINE LDL RECEPTOR IgG

Purified bovine LDL receptor (0.4 $\mu\text{g}/\text{lane}$) and octyl glucoside extract of chicken oocyte membranes (35 μg protein/lane) were subjected to electrophoresis on 4.5-18% SDS-polyacrylamide gradient gels under non-reducing conditions, followed by transfer to nitrocellulose and immunoblotting as described in Chapter II.J. Incubations contained 10 $\mu\text{g}/\text{ml}$ of anti-bovine LDL receptor IgG (*Immune*) or pre-immune rabbit IgG (*Control*). To detect bound IgG, ^{125}I -labelled goat anti-rabbit IgG (0.5 $\mu\text{g}/\text{ml}$; specific activity, ~ 1000 cpm/ng) was used. Exposure to XAR-5 film was for 12 hr at 23 °C. M_r standards are indicated.

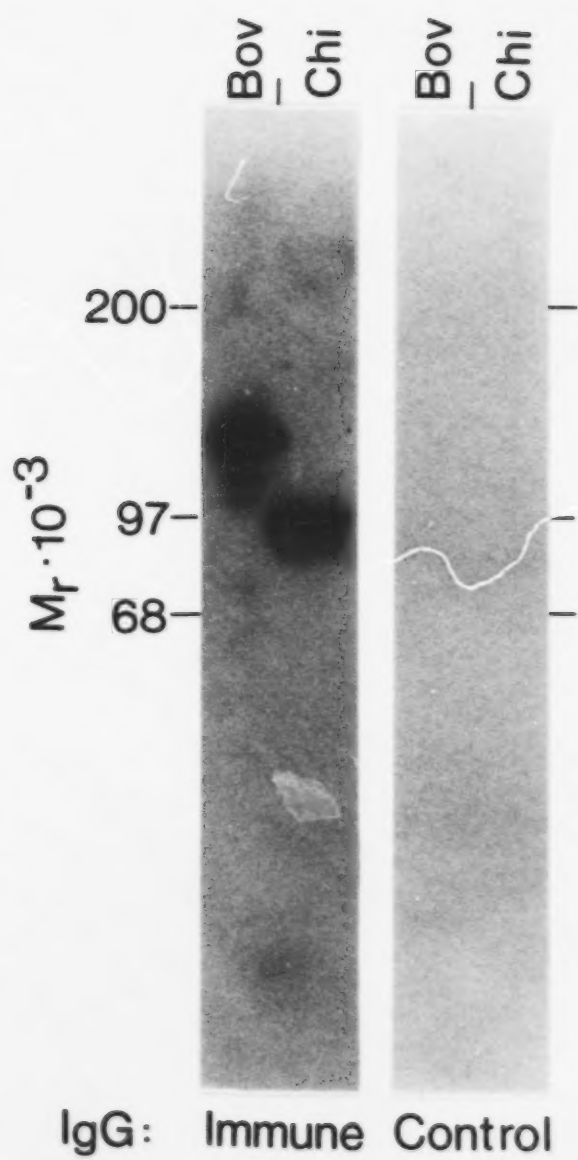


FIGURE III 7

ENDOGLYCOSIDASE DIGESTIONS OF PARTIALLY PURIFIED CHICKEN OOCYTE 95 kDa VLDL RECEPTOR

Aliquots containing 20 μ g of VLDL-Sepharose 4B eluate were incubated with various endoglycosidases as described in Chapter II.K. Samples were then subjected to non-reducing SDS-polyacrylamide gel electrophoresis on a 4.5-18% gradient gel, followed by electrophoretic transfer to nitrocellulose. The nitrocellulose strips were incubated with laying hen ^{125} I-VLDL (12 μ g/ml, 90 cpm/ng). Exposure to Kodak AR film was for 6 hr at 23 °C. *Lanes A and D*, no enzyme; *lane B*, 0.2 U endoglycosidase H; *lane C*, 0.2 U endoglycosidase F; *lanes E and G*, no enzyme; *lane F*, 300 U N-glycanase; *lanes H and L*, no enzyme; *lane I*, 0.2 U neuraminidase; *lane J*, 0.2 U neuraminidase followed by 4 mU O-glycanase; *lane K*, 4 mU O-glycanase.

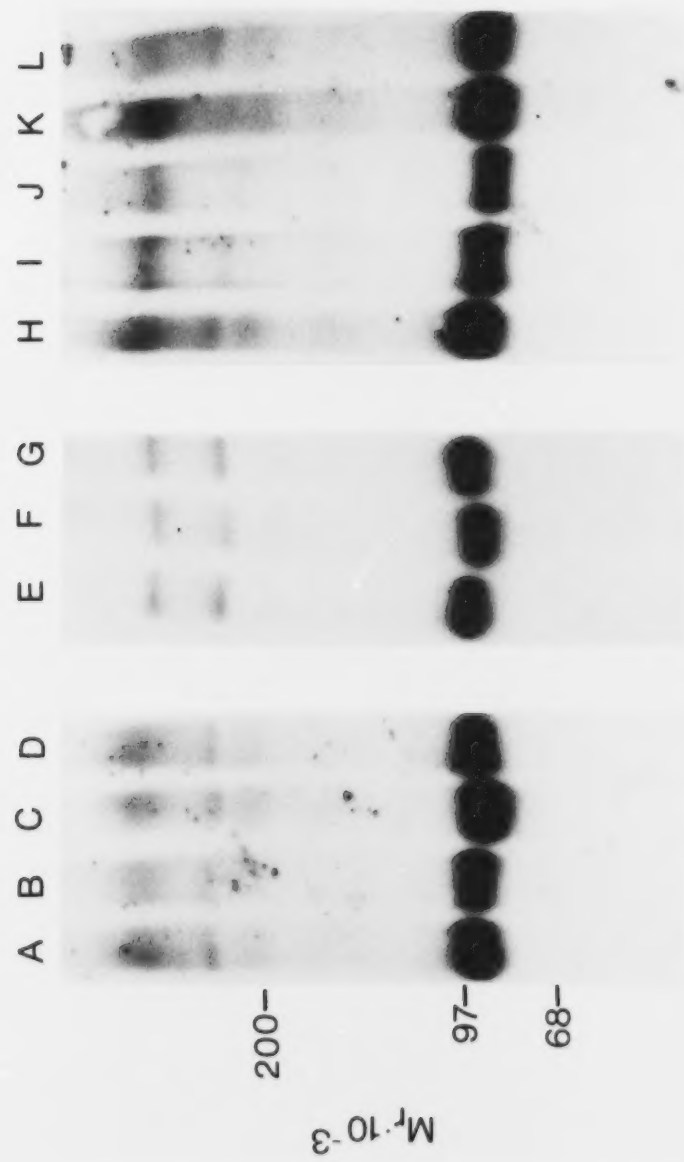
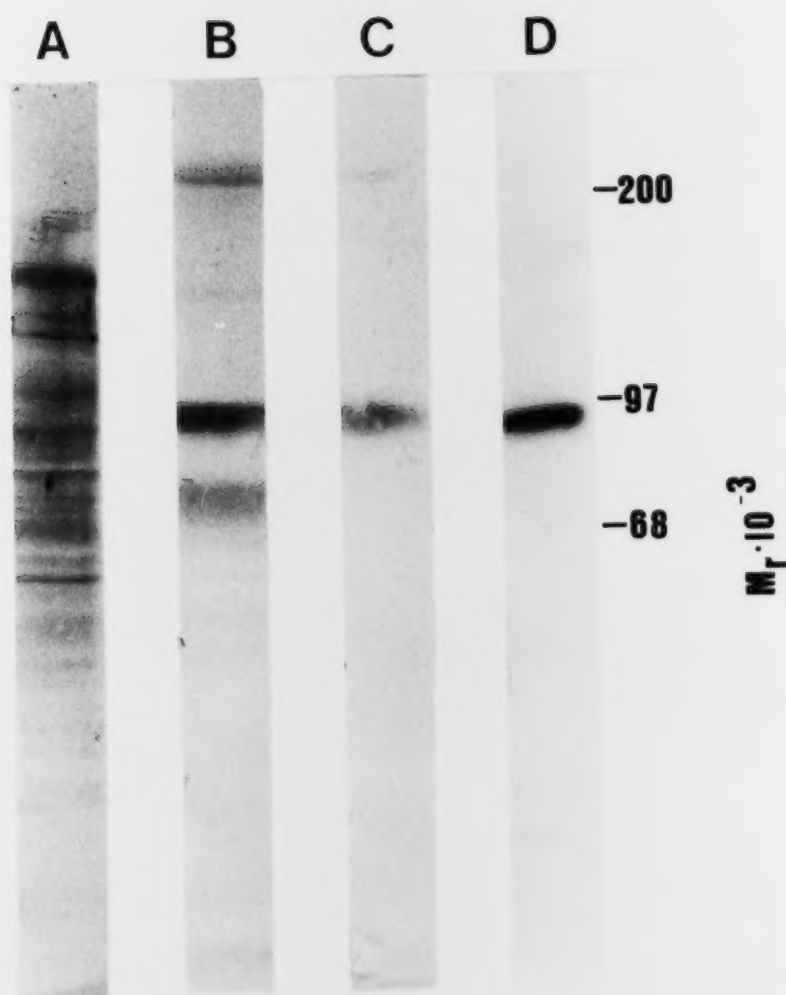


FIGURE III 8

LECTIN BLOTTING OF OOCYTE MEMBRANE OCTYL GLUCOSIDE EXTRACTS

Aliquots containing 20 μg of octyl glucoside extract of chicken oocyte membranes (*lanes A, B and D*) and 5 μg of VLDL-Sepharose 4B (*lane C*) were separated via non-reducing SDS-polyacrylamide gel electrophoresis on 4.5-18% gradient gels, followed by electrophoretic transfer to nitrocellulose. The nitrocellulose strips were then incubated with 1 $\mu\text{g}/\text{ml}$ peroxidase-labelled Concanavalin A (*lane A*) or 1 $\mu\text{g}/\text{ml}$ *Bandeiraea simplicifolia* (*lanes B and C*) followed by visualization with the substrate, 4-chloro-1-naphthol, as described in Chapter II.L. *Lane D* shows a ligand blot with ^{125}I -VLDL (12 $\mu\text{g}/\text{ml}$, 90 cpm/ng). The strip was exposed to Kodak AR film for 4 hr at 23 °C.



CHAPTER IV

CHARACTERIZATION OF A MULTIFUNCTIONAL TRANSPORT PROTEIN: THE AVIAN 95 kDa VLDL/VTG RECEPTOR²

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IV.A INTRODUCTION

In the last chapter, biochemical evidence suggested that a 95 kDa membrane glycoprotein mediated VLDL transport into the oocyte. At the same time that these studies were conducted, Stefano Stifani, a graduate student in the laboratory, was searching for a putative VTG receptor. He had shown through similar approaches that VTG also bound in a high affinity, specific and saturable fashion to an oocyte 95 kDa protein. Ligand blotting experiments showed that ^{125}I -VLDL bound to a 95 kDa protein from detergent solubilized oocyte membranes and that this specific binding could be displaced by an excess of unlabelled VLDL or unlabelled VTG. An excess of unlabelled VTG or unlabelled VLDL could also compete for ^{125}I -VTG binding to the 95 kDa receptor [230]. We became intrigued by the possibility that a single 95 kDa oocyte membrane glycoprotein may transport both of the major ligands VLDL and VTG into the oocyte. Kozo Hayashi had characterized the somatic 130 kDa LDL receptor from chicken fibroblasts in the laboratory, and had shown that this protein is unable to bind apo E-containing β -VLDL from rabbit [228]. Interestingly, this same ligand bound to the 95 kDa receptor, and a polyclonal antibody raised against the purified bovine LDL receptor recognized the oocyte receptor but not the protein expressed in somatic tissues, as described in Chapter III. This would suggest that the 95 kDa VLDL receptor may be more closely related to the mammalian LDL receptor than its 130 kDa somatic counterpart.

A mutant strain of chicken referred to as restricted ovulator (R/O) expresses a single gene defect [231] and is unable to lay eggs. Research completed by Dr. Nimpf in our laboratory had shown that oocytes from homozygous *ro/ro* mutants lacked functional VLDL receptors [232] and VTG receptors [230]. The R/O model represents a powerful tool to independently address the function of avian lipoprotein receptors.

Considering a ligand-receptor system was under study and having a panel of polyclonal antibodies available, purification of the 95 kDa protein was sought in order to test if the pure protein bound both VLDL and VTG.

IV.B. RESULTS

IV.B.1 PURIFICATION OF THE 95 kDa PROTEIN

Initial experiments on oocyte isolation indicated that peak levels of receptor activity were isolated from oocytes 3-15 mm in diameter (data not shown). For purification experiments, smaller oocytes were collected and homogenized whole.

The 95 kDa receptor can be solubilized from oocyte membranes using a variety of detergents as shown in Figure IV 1. A Coomassie stain illustrates that CHAPS (*lane 1*), octyl glucoside (*lane 2*), Nonidet P-40 (*lane 3*) and Triton X-100 (*lane 4*) solubilize a large number of proteins from follicle membranes. A ligand blot in Panel B shows that ^{125}I -VLDL binds to a 95 kDa protein ($\blacklozenge$) from all detergent solubilized extracts. This binding is specific as a 100-fold excess of unlabelled VLDL competes for binding (Panel C). Octyl glucoside does not solubilize as many proteins as other detergents as shown in the Coomassie stain, and ligand blotting indicates that the relative amount of the 95 kDa receptor produced from octyl glucoside solubilization is also lower than other detergents. Another higher molecular weight protein ($\diamond$) binds ^{125}I -VLDL in ligand blots. This was not investigated further at this time but will be addressed later in this chapter. For quantitative analysis such as binding assays, octyl glucoside or CHAPS were used because of their high CMC. Initial purification experiments were completed using CHAPS solubilized extract in order to monitor A280. However, subsequent experiments were conducted using Triton X-100 solubilized oocyte membranes, once the chromatographic properties of the particular matrix were identified.

A rapid, two-step affinity chromatography procedure was designed to facilitate purification of the 95 kDa receptor. Triton X-100 solubilized oocyte membrane extract was applied to a VLDL-Sepharose 4B column. After washing the column in low ionic strength

buffer (buffer O), bound proteins were eluted using an ammonia gradient. This material was then applied to an Anti-receptor IgG-Sepharose 4B column. After extensive washing of the column with a buffer containing 1 M NaCl, the 95 kDa protein was isolated by elution with 0.5 M NH₄OH. Various fractions from the chromatography experiments are shown in Figure IV 2. Ligand blotting experiments (panel B) shows that ¹²⁵I-VLDL also binds weakly to the 380 kDa protein (◇) isolated from VLDL-Sepharose 4B chromatography. An immunoblot in Panel D illustrates that the IgG utilized for purification binds to a higher M_r band (◄), which is distinct from the 380 kDa band and probably represents receptor dimer [225].

Purified 95 kDa receptor displays a different mobility in the presence of reducing agents in SDS-polyacrylamide gel electrophoresis. Figure IV 3 illustrates that under reducing conditions the protein migrates at 110 kDa (*lane C*) indicating that internal disulfide(s) bonds are present. This suggests that a cysteine-rich repeat structure may be found in the ligand binding domain of the oocyte receptor like the mammalian LDL receptor [139], which migrates at 130 kDa under non-reducing conditions and 160 kDa in the presence of reducing agent [151]. The oocyte VLDL receptor displays different mobility in crude extract (*lane B*) (▷) and in a purified state (*lane C*) which is most likely attributed to different buffer conditions in the two samples, as no proteolysis is observed in *lane C*.

Mr. Stifani had been investigating the receptor for vitellogenin (VTG), another serum protein transported into the oocyte. His studies suggested that VTG interacted with a 95 kDa protein. We were interested to determine if the pure 95 kDa protein bound VTG. A ligand blot in Figure IV 4 illustrates that both ¹²⁵I-VLDL and ¹²⁵I-VTG bind to the 95 kDa receptor. This interaction was competed by a 100-fold excess of unlabelled VLDL or unlabelled VTG for either labelled ligand. Note that binding is abolished in the presence of unlabelled VTG, whereas slight binding is observed with unlabelled VLDL. Although the protein concentration of each unlabelled ligand is similar, the effective ligand concentration of VLDL would be lower due to the presence of apo VLDL-II.

IV.B.2. IMMUNOCYTOCHEMISTRY

The anti 95 kDa antibody was also used for immunocytochemical localization of the VLDL/VTG receptor in collaboration with Dr. E. Sanders as shown in Figure IV 5. In order to demonstrate the distribution of the 95 kDa receptor among follicular cells, two approaches were taken. A contiguous sheet consisting of granulosa cells attached to the basement membrane on one side and bearing the perivitelline layer with adhering oocyte plasma membrane and underlying structures on the other side was isolated (Figure IV 5B). When thin sections of such preparations were incubated with affinity purified anti-95 kDa antibody followed by gold-labelled Protein A, immunoreactivity was observed in the plasma membrane region of the oocyte, with a few gold particles in the perivitelline layer, into which processes of the oocyte surface membrane are thought to extend [15] (Figure IV 5C). In the plasma membrane and underlying area, the receptor was predominantly localized to coated pits on the surface and to intracellular vesicular structures as shown in Figure IV 5D. The 95 kDa protein was undetectable within and on the surface of granulosa cells (Figure IV 5E), in agreement with previous biochemical findings demonstrating the absence of the VTG/VLDL receptor from these cells [229], as well as from fibroblasts [228].

Immunocytochemical analysis was performed on thin sections prepared from intact ovarian follicles that had been surgically denuded of the external thecal layers and subsequently fixed. The receptor was present within the electron lucent phase of endocytic compartments termed yolk spheres, which are believed to represent storage organelles for yolk proteins [183] (Figure IV 6A). The VLDL/VTG receptor was also clearly visualized in the periphery of the oocyte, primarily associated with coated pits and vesicles as observed in Figures IV 6, B and C. No significant immunoreactivity was observed in thin sections of follicles from receptor defective R/O hens (Figure IV 6 D). A consistent observation was the electron dense appearance of the contents of vesicles in the mutant as compared with normal oocytes, clearly visualized in the thin section shown in Figure

IV 6D. Incubations with pre-immune IgG resulted in the absence of gold particles in all tissue sections (data not shown). This evidence indicates that the 95 kDa protein is specifically expressed by the oocyte and absent from somatic cells, and furthermore, mediates the uptake of lipoproteins via a coated pit/coated vesicle mechanism.

IV.B.3. SEQUENCE DETERMINATION

To gain further primary structural information about the 95 kDa VLDL receptor, amino acid sequence was obtained from three tryptic fragments of the purified 95 kDa receptor by Dr. R. Aebersold. These sequences could be aligned with sequences of LDL receptors from human [139], rabbit [154], rat [233], *Xenopus* [234] and bovine [235] species as shown in Figure IV 7. Peptide I corresponds to a repeat structure, **YWTD**, found in the EGF precursor-type repeats of the LDL receptor, LDL receptor-related protein (LRP), and related proteins corresponding to the consensus sequence of 5 repeated modules of approximately 45 residues each [140]. Peptide II also matches well with a highly conserved region in the EGF precursor domain in close proximity to the last of the 5 YWTD modules. The most interesting sequence obtained was found in Peptide III which corresponds to the NPXY internalization sequence discovered in membrane receptors such as the LDL and EGF receptors [165]. The avian sequence **NFDNPVY** is identical to the sequences from all other known LDL receptors, suggesting that the oocyte protein plays an integral role in receptor mediated oocyte growth.

IV.B.4. QUANTITATIVE BINDING ANALYSIS

The ligand blot in Figure IV 4 provided evidence that the purified 95 kDa receptor bound not only apo B-containing ligands, but VTG as well. This possibility was further examined by completing a number of solid phase binding assays. Saturation curves illustrating the binding of VLDL, LDL and VTG are shown in Figures IV 8-10, respectively. All ligands bind to the purified 95 kDa receptor with high affinity: VLDL, K_d of 2.6 $\mu\text{g/ml}$; LDL, K_d of 4.6 $\mu\text{g/ml}$; and VTG, K_d of 2.8 $\mu\text{g/ml}$. An excess of unlabelled ligand or 10 mg/ml suramin (for VTG) competes for specific binding. VTG

displays a propensity to aggregate at high concentrations, so suramin was utilized as an alternative competitor of specific ^{125}I -VTG mediated binding [64]. Assuming M_r values of 950 000 for the protein moiety of VLDL [236], 512 000 for apo B of LDL and 440 000 for the circulating VTG dimer [53], the K_d values are 2.7 nM (VLDL), 9.0 nM (LDL) and 6.4 nM (VTG).

Unfortunately, the specific activity of the receptor preparation cannot be accurately assessed by this assay due to lack of retention of a portion of the receptor-ligand complexes on the cellulose acetate filters, as has previously been demonstrated [138]. Calculated $B_{\max}$ values are 14.4 μg VLDL bound/mg; 6.9 μg LDL bound/mg; and 2.2 μg VTG bound/mg. The difficulty with this assay lies in the proper selection of a membrane filter that allows free ligand, such as VLDL particles 38 nm in size to pass through, but retains ligand-receptor aggregates.

To address the stoichiometry of the ligand-receptor interaction, a double labelling experiment was conducted. Both ligand and receptor were independently labelled with Na^{125}I . As shown in Figure IV 11, ^{125}I -labelled receptor was added to increasing concentrations of unlabelled VLDL to determine the amount of receptor retained on the filter. In another set of incubations, the same dilutions of ^{125}I -VLDL were added to a fixed amount of ^{125}I -receptor. A saturation curve was obtained and a K_d value of 3.8 $\mu\text{g}/\text{ml}$ was observed, illustrating that the receptor was not significantly altered by iodination. When an excess of unlabelled VLDL was added to the double label incubation, only non-specific binding was observed (data not shown). Approximately 35% of the receptor was retained on the filters in the absence of ligand in the presence of up to 40 $\mu\text{g}/\text{ml}$ VLDL. From these data and knowledge of the specific activities of the ligand and receptor preparations, the amount of VLDL bound to the receptor at saturation was calculated to be 7 mg VLDL/mg receptor. Assuming an aggregate M_r of 950 000 for apo B and apo VLDL-II, and a protein M_r of the oocyte receptor of 90 000, 0.7 mol VLDL bound/mol receptor.

The binding of VTG to the 95 kDa receptor was also evaluated in a double label binding assay as shown in Figure IV 12. Approximately 35% of radiolabelled receptor was retained on the filter in the presence of 50 $\mu\text{g/ml}$ unlabelled VTG. Saturation was achieved by the addition of ^{125}I -VTG to ^{125}I -receptor. Assuming a VTG dimer weight of 440 kDa, the value of 6.0 mg VTG bound/mg receptor converts to 1.3 mol VTG bound/mol receptor.

IV.B.5. COMPETITIVE BINDING

The binding studies described have provided concrete evidence that two diverse ligands, apolipoprotein B and vitellogenin, bind in a high affinity, saturable manner to a 95 kDa oocyte glycoprotein. In order to determine the specificity of this interaction, a number of competitive binding assays were completed as shown in Figures IV 13-15. A fixed subsaturating concentration of labelled ligand is selected, and increasing concentrations of unlabelled ligands are added to the assay mixture containing a constant aliquot of pure receptor suspension. All other steps are performed as previously described in saturation binding experiments.

Specific VLDL binding can be competed by either unlabelled VLDL or unlabelled VTG as shown in Figure IV 13. The competition by VTG does not mirror that of VLDL, probably due to its aggregation effects, as at 250 $\mu\text{g/ml}$ the value is 62% of control (data not shown). Lysine residues have been shown to be vital for ligand interaction with the human LDL receptor since reductively methylated apo B or apo E was unable to bind to the receptor protein [134]. Chicken VLDL that has undergone reductive methylation is unable to compete for ^{125}I -VLDL binding, as is reductively methylated VTG (data not shown). Chicken HDL also shows only slight competition.

These results were supported in Figure IV 14, where it is observed that not only unlabelled LDL, but also unlabelled VLDL and unlabelled VTG compete for rooster ^{125}I -LDL binding. Reductively methylated LDL shows competition at higher concentrations indicating that the methylation reaction may not have been complete.

Unlabelled VTG is the only ligand that competes for specific ^{125}I -VTG binding (Figure IV 15). Neither unlabelled VLDL or unlabelled reductively methylated VLDL (data not shown) competes for ^{125}I -VTG binding. Lysine residues are also vital for the interaction of VTG with the pure 95 kDa receptor, as reductively methylated VTG is also unable to compete for ligand binding. Rooster HDL shows partial displacement of ^{125}I -VTG binding, but up to 250 $\mu\text{g/ml}$ of competitor less than 25% inhibition was observed. This may indicate that slight contamination of HDL with apo B or VTG may have occurred. However, the degree of competition does not increase at higher indications probably indicating that the observed competition may arise from some artifact of the binding assay due to mixed ligand interactions.

Previous studies completed by Dr. Hayashi in our laboratory indicated that apolipoprotein E-containing β -VLDL isolated from a cholesterol-fed rabbit binds to the 95 kDa VLDL/VTG receptor, but not to the 130 kDa fibroblast receptor [228]. In order to test this possibility further, Dr. Steyrer in our laboratory prepared dimyristoylphosphatidylcholine-reconstituted human apo E3/3 particles. The ability of this substrate to compete for ^{125}I -VLDL binding was tested. Figure IV 16 shows that human apo E3/3 competes for chicken ^{125}I -VLDL interaction to the purified 95 kDa receptor. Unlabelled HDL, as before, is unable to compete.

IV.B.6. PROTEASE DIGESTIONS

Since both VLDL and VTG bind to the 95 kDa receptor, but display different ligand specificity, protease digestions were conducted in order to isolate discrete ligand binding domains of the receptor. A battery of different proteases was tested and some of the data are shown in Figure IV 17. Ligand blotting of digests showed that ^{125}I -VTG (top panel) and ^{125}I -VLDL (bottom panel) recognized the same fragments. The only differential pattern of digestion was revealed in tryptic digests (1:1000, 18 hr, 37 ° C) in which ^{125}I -VTG bound to a 50 kDa fragment and a 70 kDa fragment reacted with ^{125}I -VLDL (data not shown). Competition with unlabelled ligands corroborated the quantitative

binding analysis completed earlier. VTG binding is efficiently competed only by unlabelled VTG; a 100-fold excess of unlabelled VLDL shows a small degree of competition. Either unlabelled VLDL or unlabelled VTG competed for ^{125}I -VLDL binding. Extending the digestions for longer periods or increasing the enzyme:substrate ratios generated smaller fragments as determined by Coomassie staining (data not shown). However, in no case was a smaller binding fragment than 45 kDa observed for the binding of VLDL or VTG as determined by ligand blotting with any of the aforementioned proteases as well as Pronase and Proteinase K.

IV.C. DISCUSSION

Previous reports in the literature had provided morphological [18], [183] or biochemical evidence for receptors for VLDL [186], [187] and VTG [237]. Preliminary evidence from ligand blotting, antibody inhibition of ligand binding and studies with the restricted ovulator hen suggested that a 95 kDa protein was responsible for the uptake of the two major estrogen induced lipoproteins, VLDL and VTG [230]. The experiments described within this chapter provide the first comprehensive examination of the receptor. Binding assays have revealed that a number of ligands bind to the pure 95 kDa receptor. Protease digestion experiments show that ^{125}I -VLDL and ^{125}I -VTG not only bind to the same region of the receptor, but also to identical fragments. Immunolocalization of the receptor to coated pits and intracellular vesicular structures and the presence of the NPVY internalization sequence augments the biochemical evidence in describing the vital role that this membrane protein plays in receptor mediated ligand delivery and oocyte growth.

Ability to isolate the 95 kDa receptor varies throughout growth of the oocyte. It was discovered that peak levels of receptor activity were found in smaller oocytes below 15 mm in size. It is unclear why large oocytes do not have higher amounts of intact receptor. Ghost membranes were prepared from larger oocytes and perhaps the plasma membrane is lost during this procedure. Alternatively, cathepsin D is found in the oocyte during later

stages of oocyte growth and may act on the receptor at that time or during membrane isolation.

Membranes were prepared from oocytes ranging from 3-15 mm in size, and membrane proteins were solubilized with a variety of detergents; no selective solubilization of the 95 kDa receptor was observed with Triton X-100, Nonidet P-40, octyl glucoside or CHAPS. Pure receptor was obtained by consecutive chromatographic steps over VLDL-Sepharose 4B and anti-receptor IgG-Sepharose 4B. Alternatively, yolk VLDL coupled to CNBr-activated Sepharose 4B enriched the same two proteins as observed in Figure IV 2. A number of minor bands showed non-selective interaction with the VLDL-Sepharose matrix. As the gel was used repeatedly, the number and amount of these co-purifying proteins decreased concomitantly, indicating that non-specific interaction with the lipid components of the coupled VLDL particle had occurred. Chromatography in 0.2% Triton X-100 selectively extracted lipid from VLDL. It was interesting to observe that DEAE-Cellulose 52 chromatography at pH 6.0 enriched the same two proteins as did VLDL-Sepharose 4B (data not shown).

A polyclonal antibody was obtained against 95 kDa protein eluted from a SDS-polyacrylamide gel, after the eluate had been dialyzed against 0.2% Triton X-100. Two previous attempts to isolate an antibody against SDS-eluted 95 kDa receptor had been unsuccessful, indicating that SDS may abolish antigenic domains of the protein. Pure 95 kDa receptor was obtained from anti-receptor IgG-Sepharose 4B chromatography after extensive washing of the matrix with a buffer containing 1 M NaCl. Active 95 kDa receptor could be eluted from both columns by dilute NH_4OH , as has been reported for the mammalian LDL receptor [138]. Ligand binding to the oocyte receptor under sulfhydryl reducing conditions does not occur, indicating that a disulfide-rich ligand binding domain is probably found on this protein, which is supported by the migration of this protein at 110 kDa under reducing conditions in SDS-polyacrylamide gel electrophoresis experiments.

High affinity binding of VLDL, LDL and VTG to the pure 95 kDa receptor was noted. K_d values were of higher affinity than previously reported for the binding of VLDL (13-14 $\mu\text{g/ml}$) [224], LDL (10-11 $\mu\text{g/ml}$) [224], and VTG (96 $\mu\text{g/ml}$) [64] to octyl glucoside-solubilized oocyte membrane preparations. This probably reflects ligand binding to purified receptor free of adhering endogenous yolk proteins.

A drawback of the filtration assay is the inability to directly assess the specific activity of the receptor preparation due to incomplete retention of ligand-receptor complexes on 0.45 μm cellulose acetate filters. ^{125}I -VLDL and ^{125}I -LDL suspensions were filtered through Millipore HA 0.45 μm disposable membrane filter units, but monodisperse VTG was obtained via filtration with the same cellulose acetate filter as used in the assay obtained from Advantec or Schleicher and Schuell. An appropriate membrane filter must be selected that allows free monodisperse labelled ligand to pass through while receptor-ligand complexes are retained on filtration. It is challenging to find a suitable filter for the large 38 nm VLDL particle. Cellulose acetate filters (0.45 μm) were utilized for this assay. Analysis of the B_{max} values from the binding experiments show that the values fall as the relative size of the ligand decreases: (VLDL, B_{max} of 14.4 $\mu\text{g/mg}$), (LDL, B_{max} of 6.9 $\mu\text{g/mg}$) and (VTG, B_{max} of 2.2 $\mu\text{g/mg}$) indicating that a smaller proportion of the ligand-receptor complexes are retained in each case. A double labelling experiment was conducted for VLDL and VTG to analyze the stoichiometry of ligand binding. Labelled receptor preparations were incubated with increasing concentrations of unlabelled ligand. In a number of experiments, retention of labelled receptor-unlabelled ligand complexes ranged from 35-40%. In the presence of labelled ligand and labelled receptor, a saturation curve was obtained over background binding. With knowledge of ligand and receptor specific activities, it was calculated that 1 mol of ligand bound per mol of receptor for VLDL and dimeric VTG. The K_d values did not change significantly indicating that iodination of the receptor did not alter binding affinity.

Basic residues must play an important role for binding of both VLDL and VTG to the 95 kDa receptor, as reductive methylation of either ligand alters recognition. This implies that there is some structural similarity between apo B and VTG. Competition experiments revealed that unlabelled VTG can compete for either ^{125}I -VLDL or ^{125}I -VTG binding, whereas unlabelled VLDL only competes for its own labelled ligand. A number of different possible structures could be considered for the ligand binding domains of the oocyte receptor. The competition data suggest that two classes of binding sites exist for VTG interaction. However, Scatchard analysis of ligand binding would argue against this possibility, as only a single, linear component is observed. There is some evidence for sequence similarity in apo B and VTG [238]. Could there be functionally equivalent binding sites on the receptor for each ligand where one location is accessible to both the large 38 nm VLDL particle and VTG, and the other site only binds VTG? A more plausible argument would suggest that VTG binds to a sub-structure of a large binding pocket in which apo B binds. There is precedence for this type of proposal, as the human LDL receptor requires repeats 2-7 of the cysteine-rich motif to bind apo B, but only repeat 5 is required to bind apo E [152], [150]. Isolation of a 95 kDa cDNA clone and further deletional and oligonucleotide-directed mutagenesis experiments represent the best approach to determining the structure and specificity of the ligand binding domain(s) of this protein.

Protease digestion of the 95 kDa receptor was investigated in order to identify the smallest possible ligand binding domain of the receptor. This approach had previously been utilized to elucidate the ligand binding core of the β -adrenergic [239], insulin [240], [241] and interleukin-2 [242] receptors. Binding of ^{125}I -VLDL and ^{125}I -VTG was to similar receptor fragments in most cases. The competition data supported solid phase experiments in which it was discovered that unlabelled VLDL was unable to compete for ^{125}I -VTG binding. At no time was a binding fragment smaller than 45 kDa observed, indicating that tertiary folding of the receptor is important for ligand binding. For

comparison, the smallest reported fragment of the human LDL receptor was 60 kDa isolated by thrombin treatment [144]. Ligand blotting experiments to receptor fragments confirm that both ligands bind to similar regions on the 95 kDa receptor.

Experimental observations had shown that the apo E-containing lipoprotein, β -VLDL, isolated from cholesterol-fed rabbits, bound to the 95 kDa receptor. Reconstituted human apo E3/3, which is the normal isoform of this apolipoprotein [121], competed for chicken ^{125}I -VLDL binding to the oocyte receptor in a binding assay. In other studies completed by Dr. Steyrer [203], he showed that VTG could compete for binding of apo E to the 95 kDa receptor, and an antibody directed against the 95 kDa receptor could inhibit colloidal gold-labelled apo E binding to the 95 kDa oocyte protein. This antibody had previously been shown to inhibit both VLDL- and VTG-binding to the 95 kDa receptor [230]. Dr. Steyrer also illustrated that the oocyte 95 kDa receptor binds apo E2/2, albeit at lower affinity. The K_d values were as follows: apo E3/3, 0.9 $\mu\text{g/ml}$; apo E2/2, 3.1 $\mu\text{g/ml}$; and rabbit β -VLDL, 1.6 $\mu\text{g/ml}$. These kinetic values were calculated by quantifying silver-stained adhering gold-labelled apo E in ligand blotting experiments [203]. Apolipoprotein E is not found in oviparous species, whereas VTG is not synthesized in mammalian species. The receptor binding sequence of apo E has been localized to a cluster of basic residues. Two regions of basic sequences are found in the lipovitellin region of VTG which mediates binding to the oocyte receptor [58]. This may suggest that apolipoprotein E evolved as a mammalian equivalent of VTG.

The oocyte 95 kDa VLDL/VTG receptor shares many biochemical properties with the somatic mammalian LDL receptor. Sequence analysis of three tryptic peptides revealed that there is primary structural similarity between the 95 kDa receptor and other LDL receptor proteins with known sequence. Peptide I corresponds to a characteristic repeat structure YWTD found in the EGF precursor region of LDL receptors, while peptide II showed 6/9 identity with another sequence from the same domain. The most fascinating sequence was localized to a NFDNPVY which is the internalization sequence known to be

responsible for the clustering of receptor proteins to coated pits of the cytoplasmic membrane. Together with the accumulated biochemical evidence the presence of this sequence verifies that the pure 95 kDa protein is a multifunctional transport receptor and is an evolutionary counterpart of the LDL receptor designed to mediate the transport of massive amounts of VLDL and VTG into the oocyte during the rapid growth phase.

The role that the 95 kDa protein plays in receptor-mediated uptake of serum components was further characterized by electron microscopy and immunocytochemistry. The receptor was visualized not only in coated organelles on and closely underneath the plasma membrane, but also in the area of yolk spheres [243], the final destination for storage of endocytosed plasma precursors [183]. Yolk spheres are membrane delimited storage structures that reach a size of 140 μm in diameter [183]. These large organelles contain VLDL particles [15], [244], [243], as well as electron dense subdroplets believed to consist of phosvitin and lipovitellin, the products of post-endocytic proteolytic processing of VTG [53]. Ferritin tracer studies illustrated that ligands reach mature yolk spheres within 5-10 min after arrival at the oocyte plasma membrane [244].

The immunocytochemical experiments confirmed that granulosa cells do not express the 95 kDa receptor. This is in agreement with the previously observed lack of accumulation of VLDL particles on the surface of granulosa cells [245]. For control experiments, immunocytochemical analysis of the mutant, non-laying, R/O hen oocyte was conducted. There was a striking absence of immunogold staining of coated structures from mutant oocyte samples. Together with the accumulated biochemical and primary sequence information, these immunocytochemical experiments provide convincing evidence that a single 95 kDa oocyte glycoprotein mediates the transport of VLDL and VTG. The uptake of these two components alone account for 95% of the yolk dry weight. Therefore, it is evident that the 95 kDa receptor is a key component of receptor-mediated oocyte growth.

FIGURE IV 1

DETERGENT SOLUBILIZATION OF CHICKEN OOCYTE MEMBRANE PROTEINS

All samples were analyzed on a 4.5-18% SDS-polyacrylamide gradient gel. The M_r standards (myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 29 kDa) were separated in the presence, and all other samples in the absence of 50 mM dithiothreitol. Panel A: *lane 1*, CHAPS membrane extract; *lane 2*, octyl glucoside membrane extract; *lane 3*, Nonidet P-40 membrane extract; and *lane 4*, Triton X-100 membrane extract. All samples contained 20 μ l of detergent solubilized oocyte membrane extract. Panels B and C: as above, except that each sample contained 10 μ l of membrane extract. Following electrophoretic transfer to nitrocellulose, the strips were incubated with 2.4 μ g/ml ^{125} I-VLDL (117 cpm/ng) in the absence (B) or presence (C) of 0.24 mg/ml unlabelled VLDL. Exposure of Kodak AR film was for 13 hr at -70°C .

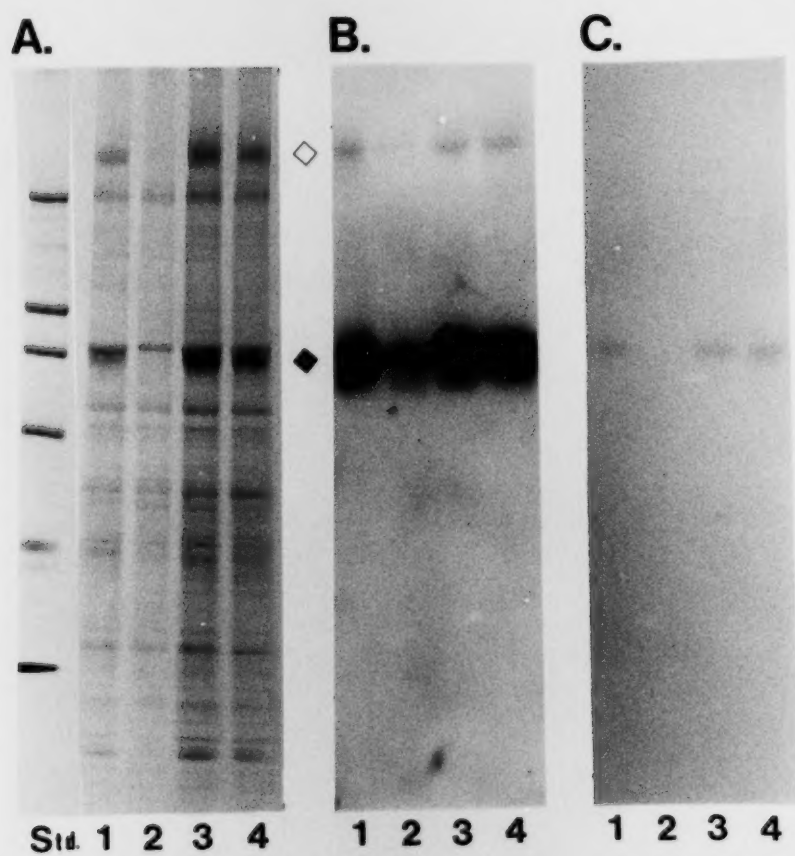


FIGURE IV 2

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE CHICKEN OOCYTE VTG/VLDL RECEPTOR AT DIFFERENT STAGES OF PURIFICATION

All samples were analyzed on a 4.5-18% SDS-polyacrylamide gradient gel. The M_r standards (myosin, 200 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 29 kDa) were separated in the presence, and all other samples in the absence of 50 mM dithiothreitol. *Panel A*: lane 1, Triton X-100 membrane extract, (20 μ g protein); lane 2, VLDL-Sepharose eluate, (9.5 μ g protein); and lane 3, immunoaffinity purified fraction, (7 μ g protein). The proteins were stained with Coomassie Blue. *Panels B-D*: Each lane contained 5 μ g of the fractions indicated in *panel A*; following electrophoretic transfer to nitrocellulose, the strips were incubated with 5.4 μ g/ml ^{125} I-VLDL (93 cpm/ng) in the absence (*B*) or presence (*C*) of 0.54 mg/ml unlabelled VLDL; or (*D*) with 25 μ g/ml of rabbit anti-receptor IgG followed by 1.7 μ g/ml of ^{125} I-Protein A (1200 cpm/ng) as described in "Materials and Methods". Exposure of Kodak AR film was for 8 hr (*panels B and C*) or 4 hr (*panel D*) at -70 °C. The position of migration of the VTG/VLDL receptor (◆) and of bands discussed in the text (◇, ◀) are indicated.

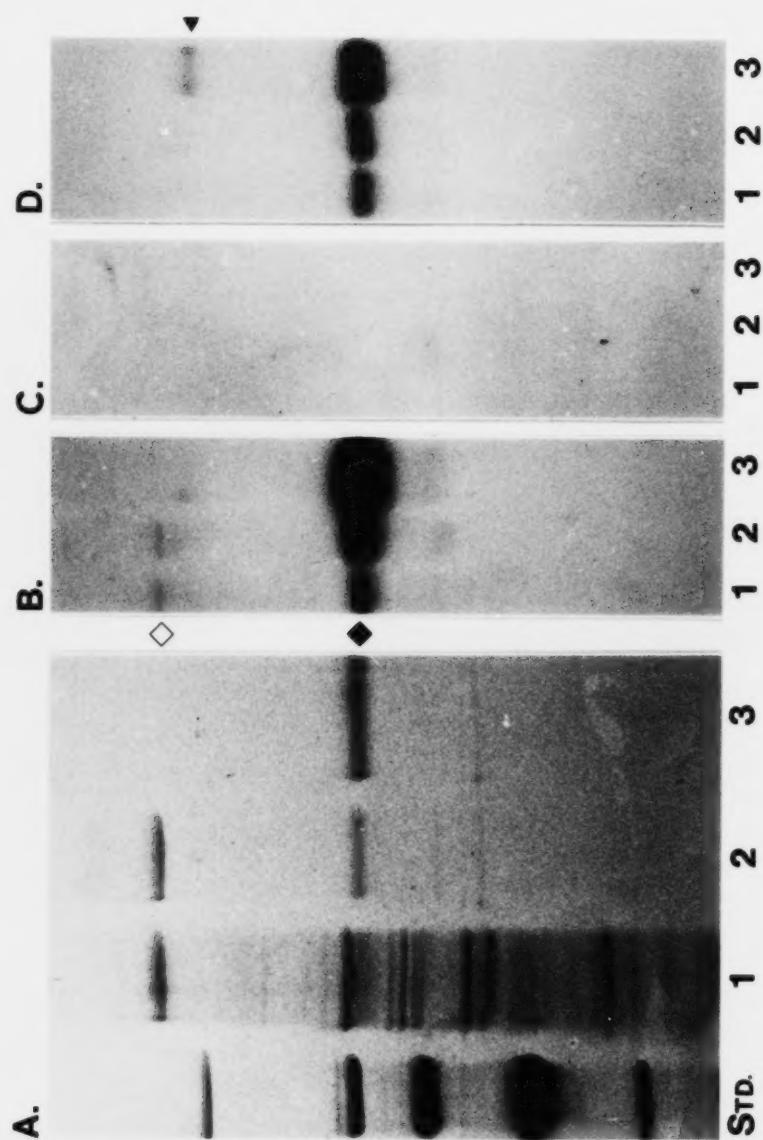


FIGURE IV 3

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE CHICKEN OOCYTE 95 kDa VLDL RECEPTOR

Samples were analyzed on a 4.5-18% SDS-polyacrylamide gradient gel in the absence (*lanes D and E*) or presence (*lanes A-C*) of 50 mM dithiothreitol and proteins were stained with Coomassie blue. *Lane A* contained the M_r standards (myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 29 kDa). *Lanes B and E*, 35 μ g of Triton X-100 solubilized oocyte membrane extract; *lane C*, 0.5 μ g of immunoaffinity purified receptor; and *lane D*, 1.5 μ g of immunoaffinity purified receptor. The location of the VLDL receptor in the detergent extract in the presence of dithiothreitol is indicated ($\triangleright$).

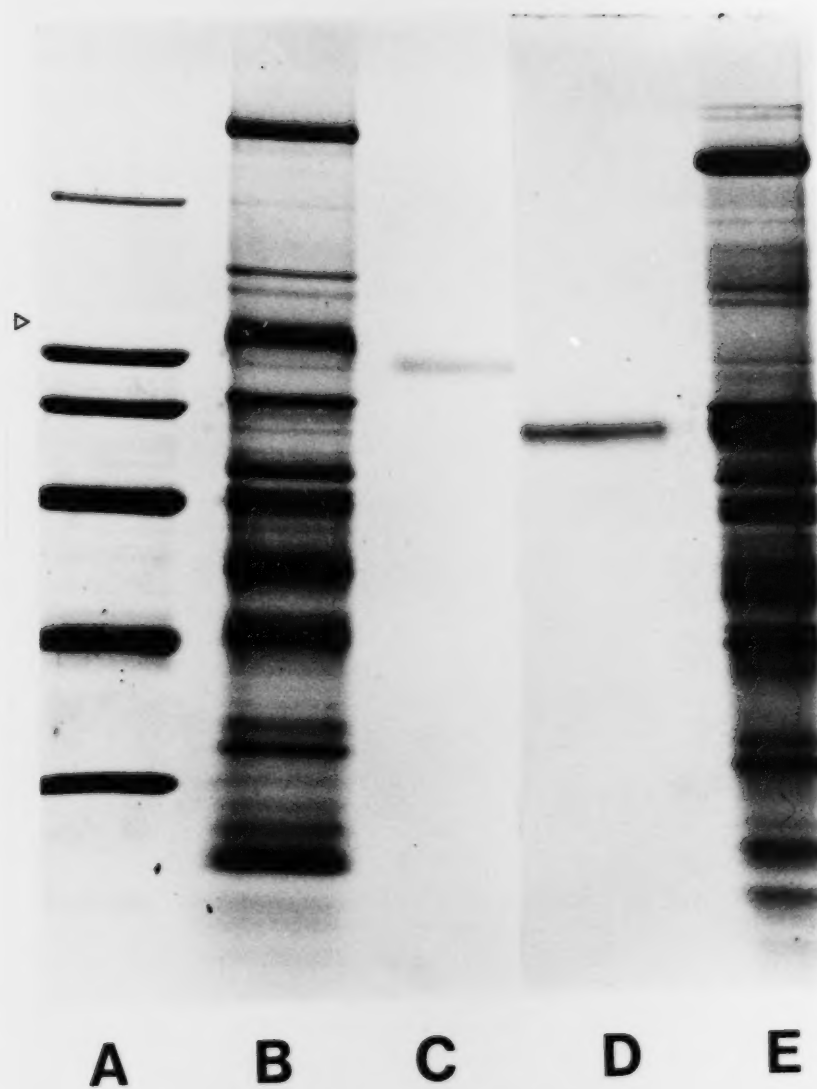


FIGURE IV 4

LIGAND BLOTTING OF AFFINITY-PURIFIED CHICKEN OOCYTE VTG/VLDL RECEPTOR

After electrophoretic separation in the absence of reducing agent and transfer to nitrocellulose, each strip containing 0.5 μ g of affinity purified material was incubated for 2 hr in buffer with the following additions: *Lanes: A-C* ^{125} I-labelled VTG at 18 μ g/ml (63 cpm/ng); *D-F*, ^{125} I-labelled VLDL at 18 μ g/ml (110 cpm/ng); *B and F*, for competition, VTG at 1.8 mg/ml was added; *C and E*, VLDL at 1.8 mg/ml was added. The autoradiograph was exposed for 24 hr (*A-C*) or 15 hr (*D-F*) at -70 °C.

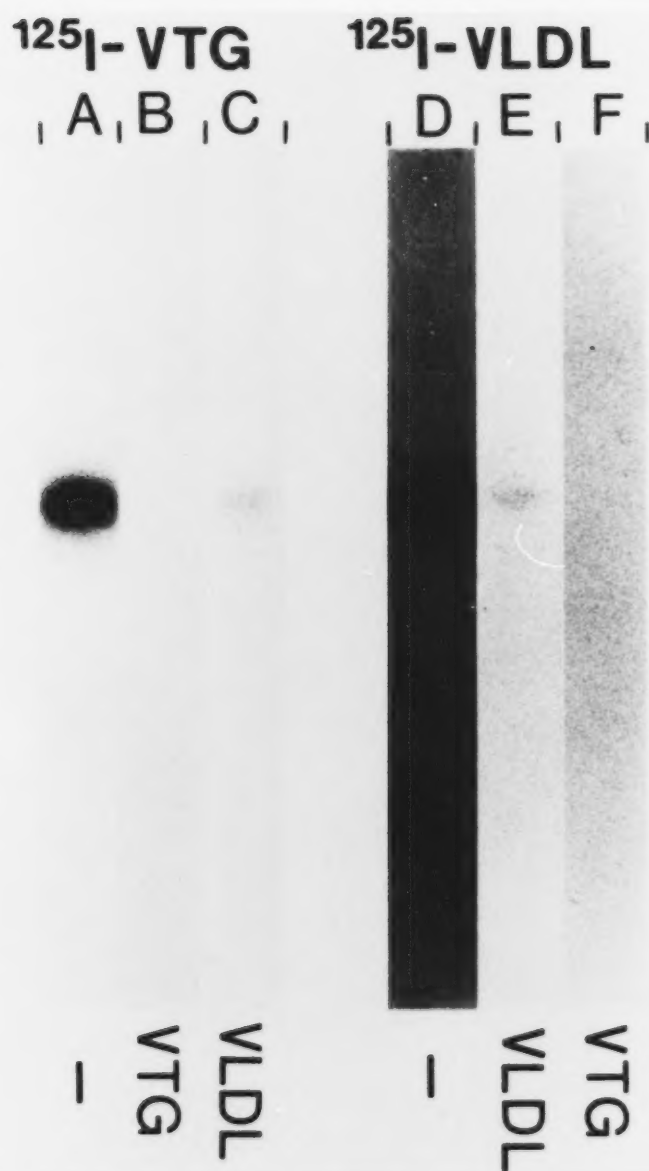


FIGURE IV 5

ULTRASTRUCTURAL IMMUNOLOCALIZATION OF THE VLDL RECEPTOR IN GRANULOSA CELL SHEETS ISOLATED FROM OVARIAN FOLLICLES

A, low power observation of an intact follicle shows the cells of the theca interna (*th*) separated by a basement membrane (*bm*) from the granulosa cell layer (*g*). The perivitelline layer (*pv*) separates the granulosa cells from the oocyte (*oo*).Yolk spheres (*y*) are visible in the oocyte. x 1 100. B, the isolated granulosa cell sheet shows an intact basement membrane (*bm*), granulosa cell layer (*g*), and perivitelline layer (*pv*). A piece of ruptured cytoplasm from the oocyte periphery (*oo*) is attached to the perivitelline layer. x1 600. C, immunogold cytochemistry of granulosa cell sheets using affinity-purified anti-receptor IgG shows labelling primarily in the oocyte remnant (*oo*), with some gold particles in the perivitelline layer (*pv*) into which processes from the oocyte extend.x3 300. D, at higher magnification, the labelling is seen to be associated with coated pits (*arrow*) and vesicles (*arrowheads*) of the oocyte remnant; x4 700. E, the granulosa cells (*g*) are not labelled by this antibody. x3 300.

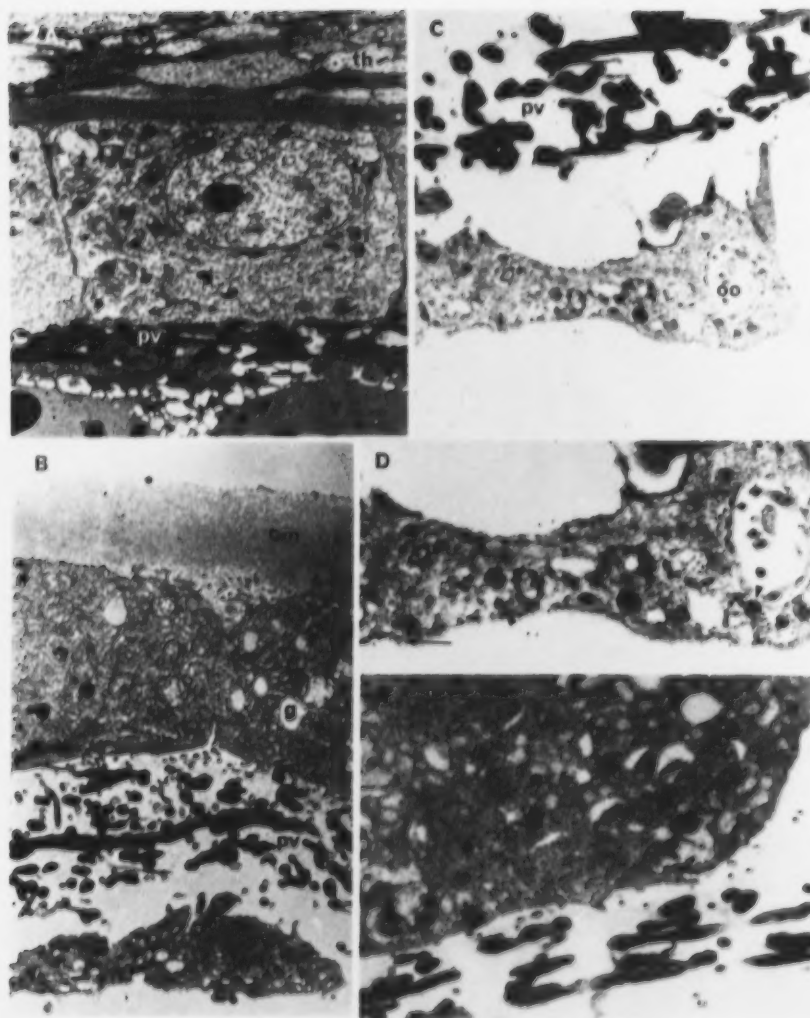


FIGURE IV 6

ULTRASTRUCTURAL IMMUNOLOCALIZATION OF THE VLDL RECEPTOR IN INTACT OVARIAN FOLLICLES

A, in the yolk spheres, the receptor is concentrated in the electron lucent phase and markedly absent from the electron dense phase. $\times 4,700$. *B* and *C*, in the periphery of the intact normal oocyte, the anti-VLDL receptor antibody binds to the receptor on the luminal surface of coated vesicles. $\times 15,400$. *D*, in contrast to normal oocytes, coated vesicles in the periphery of intact oocytes from the R/O mutant are not immunoreactive with the antibody to the VLDL receptor. Note the electron dense contents of the vesicles from the mutant oocytes in comparison with those from the normal oocytes. $\times 17,600$.

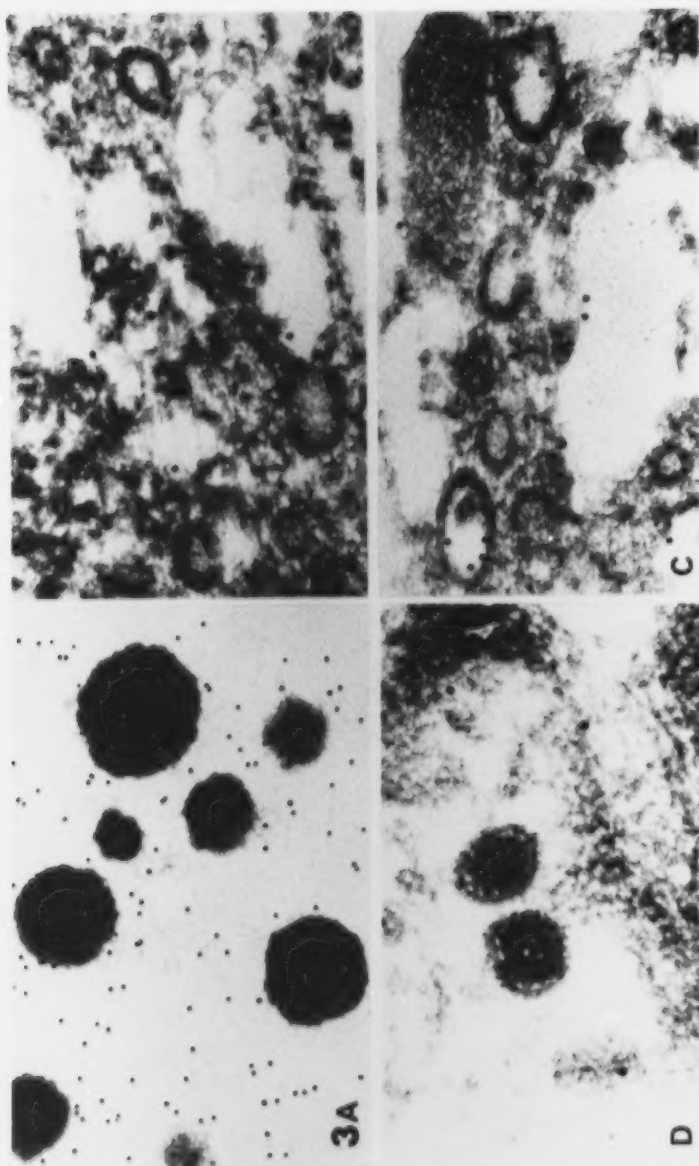


FIGURE IV 7

SEQUENCE ALIGNMENT OF REGIONS OF VARIOUS LDL RECEPTORS WITH SEQUENCED TRYPTIC PEPTIDES OF THE CHICKEN OOCYTE 95 kDa RECEPTOR

Known structurally (YWTD) or functionally (FDNPVY) significant sequences are bold-faced, and identical residues are boxed from human (hum.) [139], rabbit (rab.) [154], rat [233], *Xenopus laevis* (receptor-1 and receptor-2: Xen. 1,2) [234], and cow (bov.) [235] with chicken 95 kDa receptor (chi.). To facilitate alignment, the numbers in the bottom left corner of each box indicate the position of the respective first residues of the peptides (I, II and III) in the mature human LDL receptor [139]. The sequence of region I in the bovine LDL receptor is not available [235].

FIGURE IV 8

SATURATION CURVE AND SCATCHARD PLOT FOR THE BINDING OF CHICKEN ^{125}I -VLDL TO THE PURIFIED 95 kDa RECEPTOR

(A) Each assay tube contained the standard assay mixture (100 μl) with 7.3 μg of precipitated immunoaffinity purified receptor and the indicated concentration of radiolabelled ligand in the presence (■) or absence (●) of 3 mg/ml of unlabelled VLDL. The specific activity of the laying hen ^{125}I -VLDL was 365 cpm/ng. The amount of receptor-bound radiolabelled ligand was determined as described in Chapter II.0. High affinity binding (□) was calculated by subtracting non-specific binding (■) from total binding (●). Each data point represents the average of duplicate determinations.

(B) Binding data were analyzed by the method of Scatchard [223]. The ratio bound/free is the amount of bound ^{125}I -VLDL (μg of protein) divided by the amount of unbound protein in the reaction mixture (μg of protein).

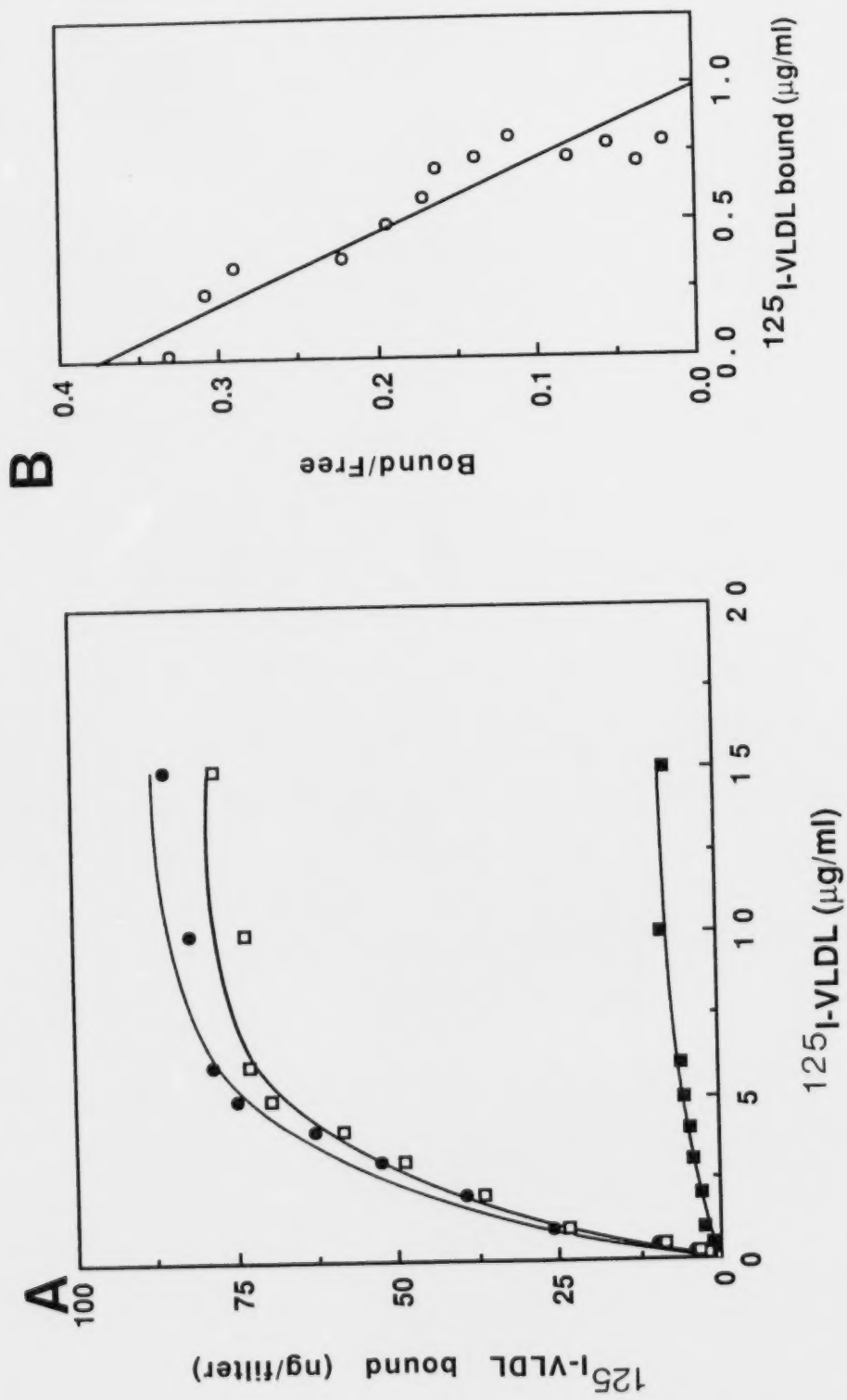


FIGURE IV 9

SATURATION CURVE AND SCATCHARD PLOT FOR THE BINDING OF CHICKEN ^{125}I -LDL TO PURIFIED 95 kDa RECEPTOR

(A) Each assay tube contained the standard assay mixture (100 μl) with 6.0 μg of precipitated immunoaffinity purified receptor and the indicated concentration of radiolabelled ligand in the presence (■) or absence (●) of 6.0 mg/ml unlabelled LDL. The specific activity of the rooster ^{125}I -LDL was 129 cpm/ng. The amount of receptor-bound radiolabelled ligand was determined as described in Chapter II.O. High affinity binding (□) was calculated by subtracting non-specific binding (■) from total binding (●). Each data point represent the average of duplicate determinations.

(B) Binding data were analyzed by the method of Scatchard [223]. The ratio bound/free is the amount of bound ^{125}I -LDL (μg of protein) divided by the amount of unbound protein in the reaction mixture (μg of protein).

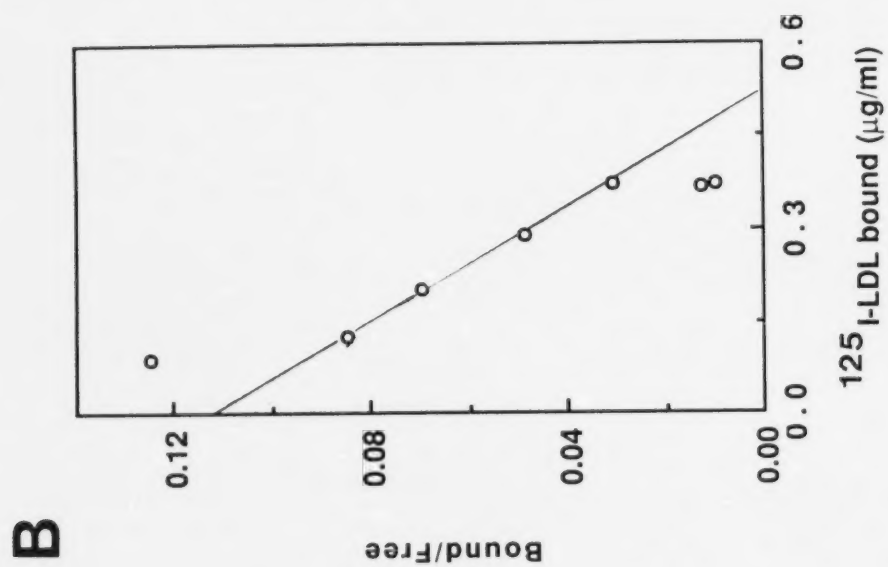
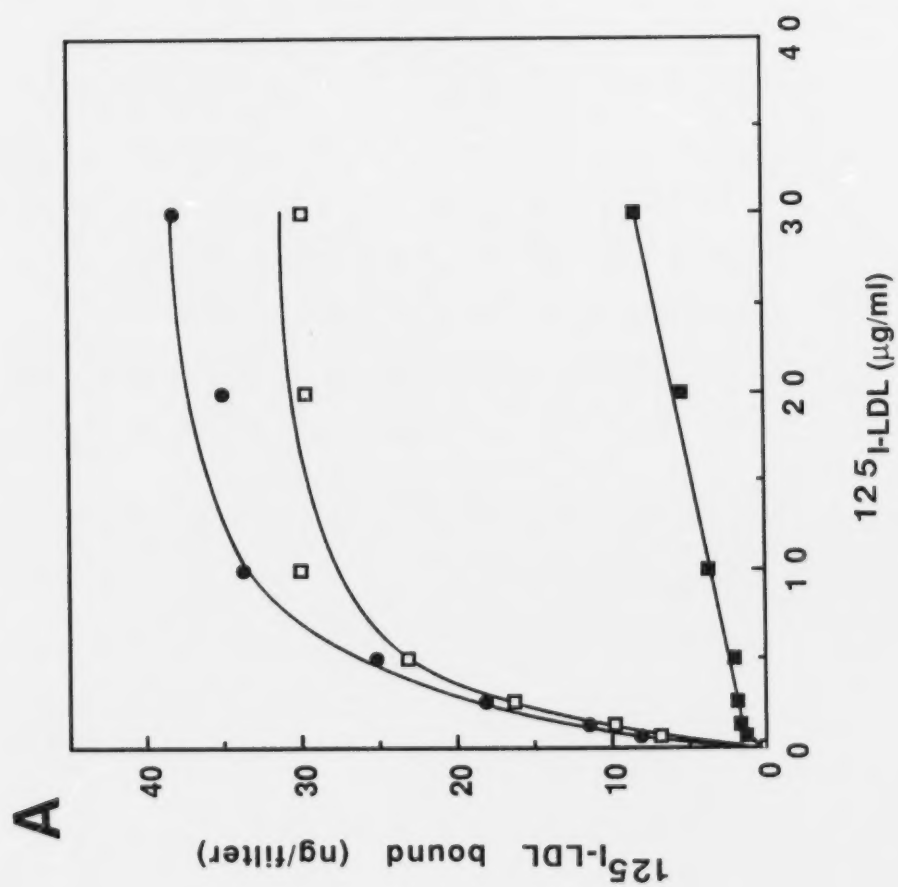


FIGURE IV 10

SATURATION CURVE AND SCATCHARD PLOT FOR THE BINDING OF CHICKEN ^{125}I -VITELLOGENIN TO PURIFIED 95 kDa RECEPTOR

(A) Each assay tube contained the standard assay mixture (100 μl) with 7.5 μg of precipitated immunoaffinity purified receptor and the indicated concentration of radiolabelled ligand in the presence (■) or absence (●) of 10 mg/ml suramin. The specific activity of the laying hen ^{125}I -VTG was 165 cpm/ng. The amount of receptor-bound radiolabelled ligand was determined as described in Chapter II.O. High affinity binding (□) was calculated by subtracting non-specific binding (■) from total binding (●). Each data point represents the average of duplicate determinations.

(B) Binding data were analyzed by the method of Scatchard [223]. The ratio bound/free is the amount of bound ^{125}I -VTG (μg of protein) divided by the amount of unbound protein in the reaction mixture (μg of protein).

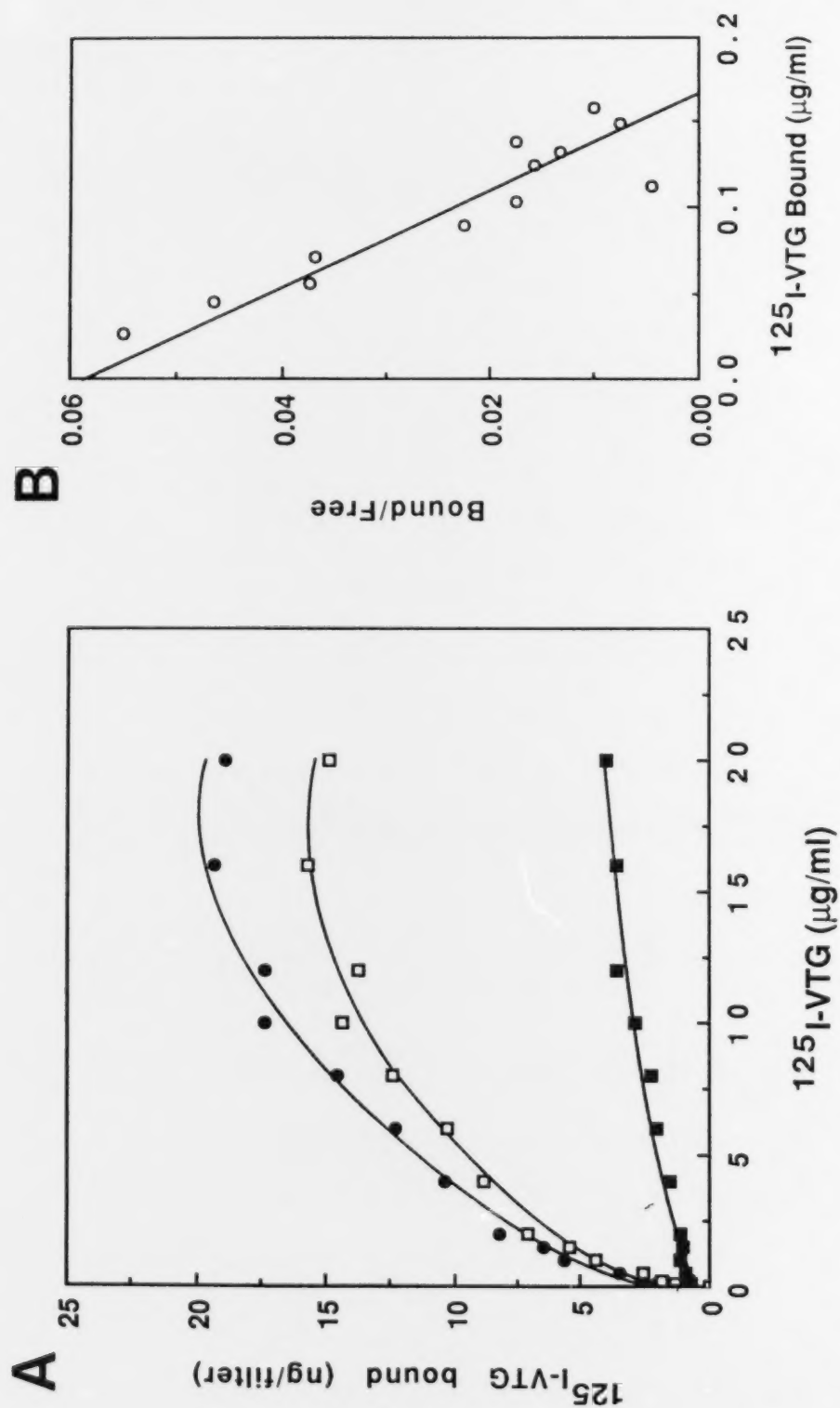


FIGURE IV 11

SATURATION CURVE FOR THE BINDING OF CHICKEN ^{125}I -VLDL TO PURIFIED ^{125}I -LABELLED 95 kDa RECEPTOR

Each assay tube (100 μl) contained 38.9 ng of acetone-precipitated ^{125}I -labelled purified 95 kDa receptor (216 cpm/ng) and the indicated concentration of either unlabelled VLDL ($\circ$) or ^{125}I -VLDL (58 cpm/ng) ($\bullet$). After incubation for 2 hr at 23 $^{\circ}\text{C}$, the total radioactivity retained by each filter ($\circ$, $\bullet$) was determined by the standard filtration assay. The data were used to calculate the amounts of ^{125}I -VLDL bound to radiolabelled receptor at each concentration of ligand ($\square$). Each data point represents the average of duplicate determinations.

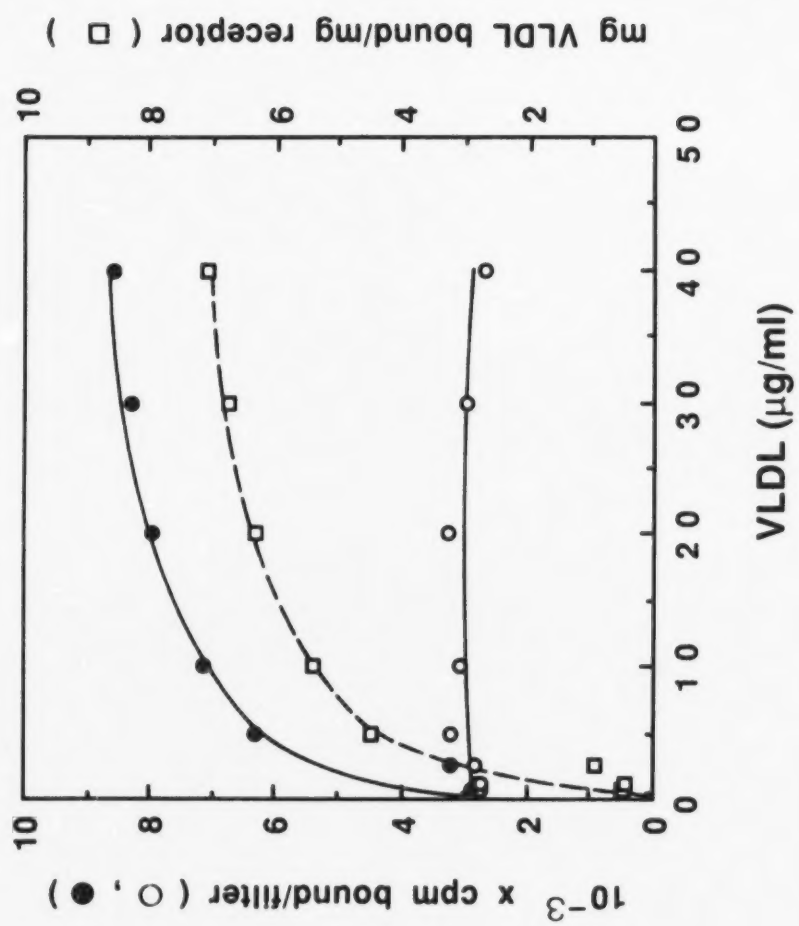


FIGURE IV 12

SATURATION CURVE FOR THE BINDING OF CHICKEN ^{125}I -VITELLOGENIN TO THE PURIFIED ^{125}I -LABELLED 95 kDa RECEPTOR

Each assay tube (100 μl) contained 22 ng of acetone-precipitated ^{125}I -labelled purified 95 kDa receptor (558 cpm/ng) and the indicated concentration of either unlabelled VTG ($\circ$) or ^{125}I -VTG (116 cpm/ng) ($\bullet$). After incubation for 2 hr at 23 $^{\circ}\text{C}$, the total radioactivity retained by each filter ($\circ$, $\bullet$) was determined by the standard filtration assay. The data were used to calculate the amounts of ^{125}I -VTG bound to radiolabelled receptor at each concentration of ligand ($\square$). Each data point represents the average of duplicate determinations.

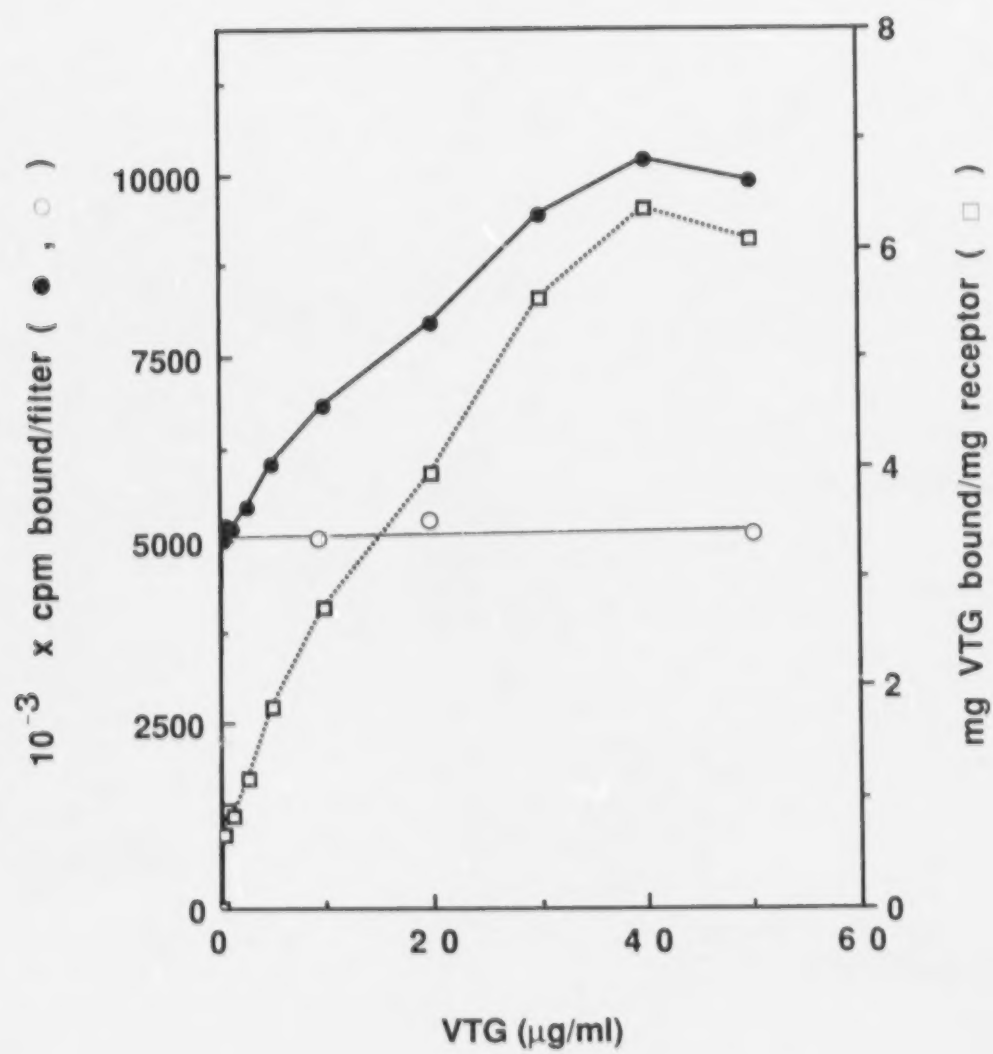


FIGURE IV 13

EFFECT OF UNLABELLED LIGANDS ON THE BINDING OF CHICKEN ^{125}I -VLDL TO PURIFIED 95 kDa RECEPTOR

Each assay tube contained the standard assay mixture (100 μl) with 15 μg of precipitated immunoaffinity purified receptor and 4 $\mu\text{g}/\text{ml}$ laying hen ^{125}I -VLDL (51 cpm/ng) in the presence of unlabelled laying hen VLDL ($\circ$), reductively methylated VLDL ($\bullet$), laying hen VTG ($\blacksquare$), or rooster HDL ($\diamond$) at the indicated concentrations. The amount of receptor-bound radiolabelled ligand was determined by filtration as described in Chapter II.O. The 100%-of-control value was 3.5 μg VLDL bound/mg protein.

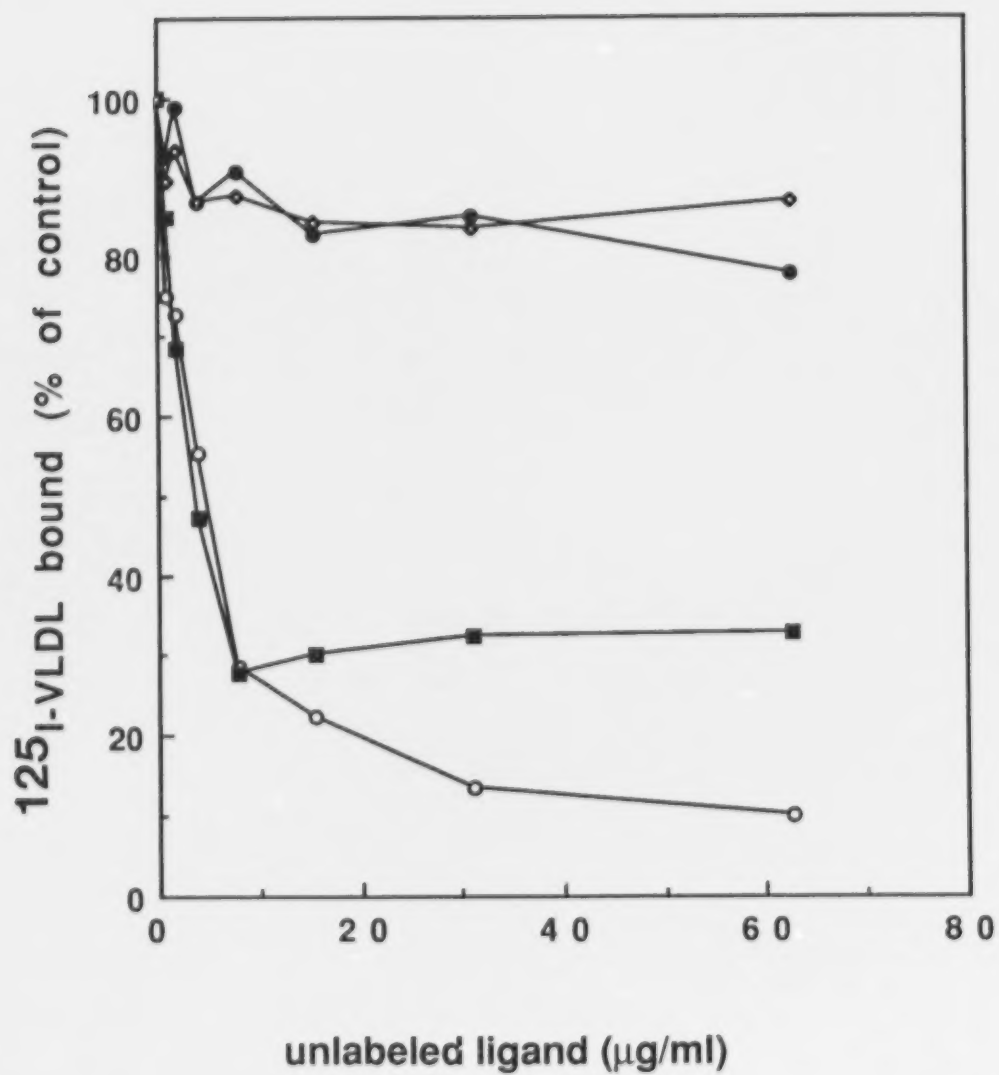


FIGURE IV 14

EFFECT OF UNLABELLED LIGANDS ON THE BINDING OF CHICKEN ^{125}I -LDL TO THE PURIFIED 95 kDa RECEPTOR

Each assay tube contained the standard assay mixture (100 μl) with 5.4 μg of precipitated immunoaffinity purified receptor and 5 $\mu\text{g}/\text{ml}$ rooster ^{125}I -LDL (145 cpm/ng) in the presence of unlabelled ligands rooster LDL (▲), reductively methylated LDL (△), laying hen VLDL (○), laying hen VTG (□) and rooster HDL (◆) at the indicated concentrations. The amount of receptor-bound radiolabelled ligand was determined by filtration as described in Chapter II.O. The 100%-of-control value was 3.8 μg LDL bound/mg protein.

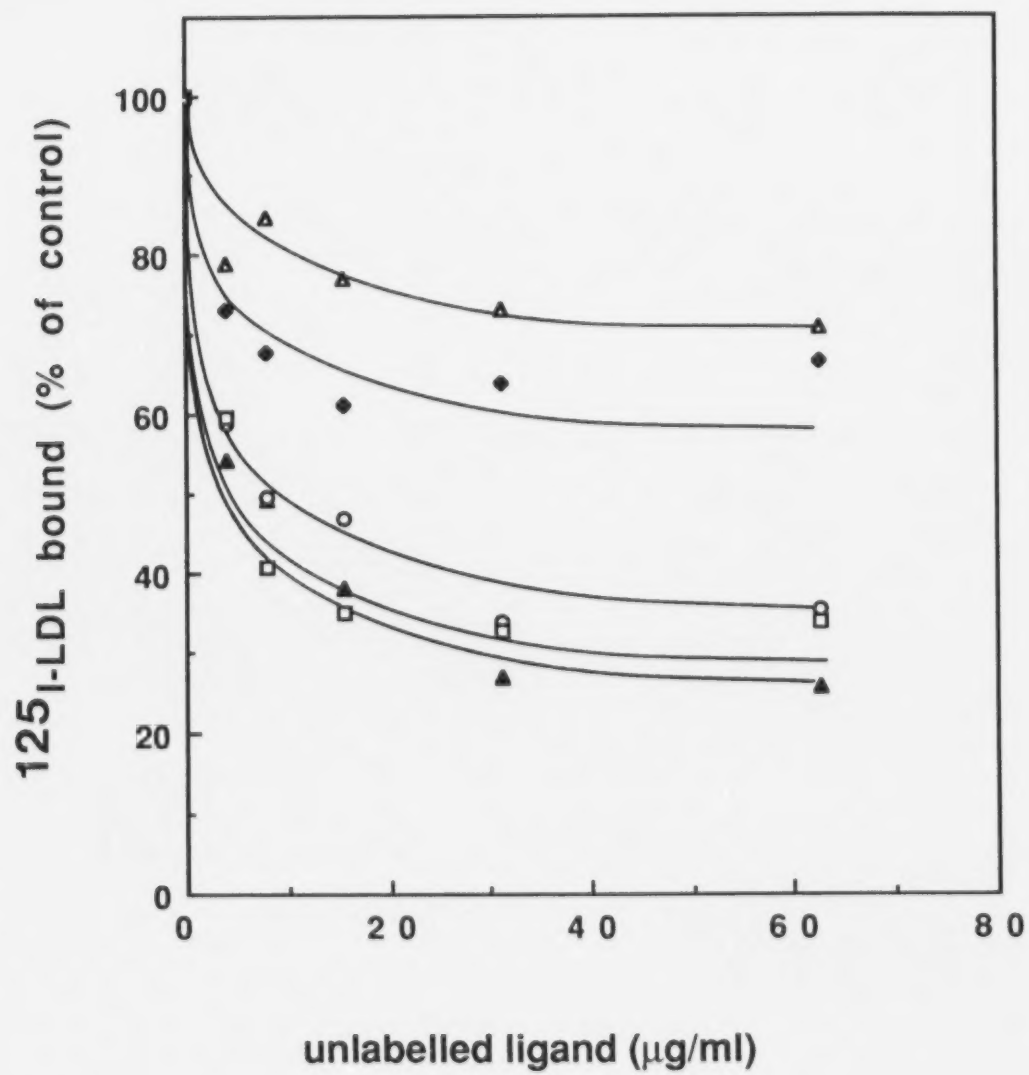


FIGURE IV 15

EFFECT OF UNLABELLED LIGANDS ON THE BINDING OF CHICKEN ¹²⁵I-VITELLOGENIN TO THE PURIFIED 95 kDa RECEPTOR

Each assay tube contained the standard assay mixture (100 μ l) with 13 μ g of precipitated immunoaffinity purified protein and 3 μ g/ml laying hen ¹²⁵I-VTG (151 cpm/ng) in the presence of unlabelled laying hen VTG ($\square$), reductively methylated VTG ($\blacksquare$), laying hen VLDL ($\circ$), or rooster HDL ($\diamond$) at the indicated concentrations. The amount of receptor-bound radiolabelled ligand was determined by filtration as described in Chapter II.O. The 100%-of-control value was 2.9 μ g VTG bound/mg protein.

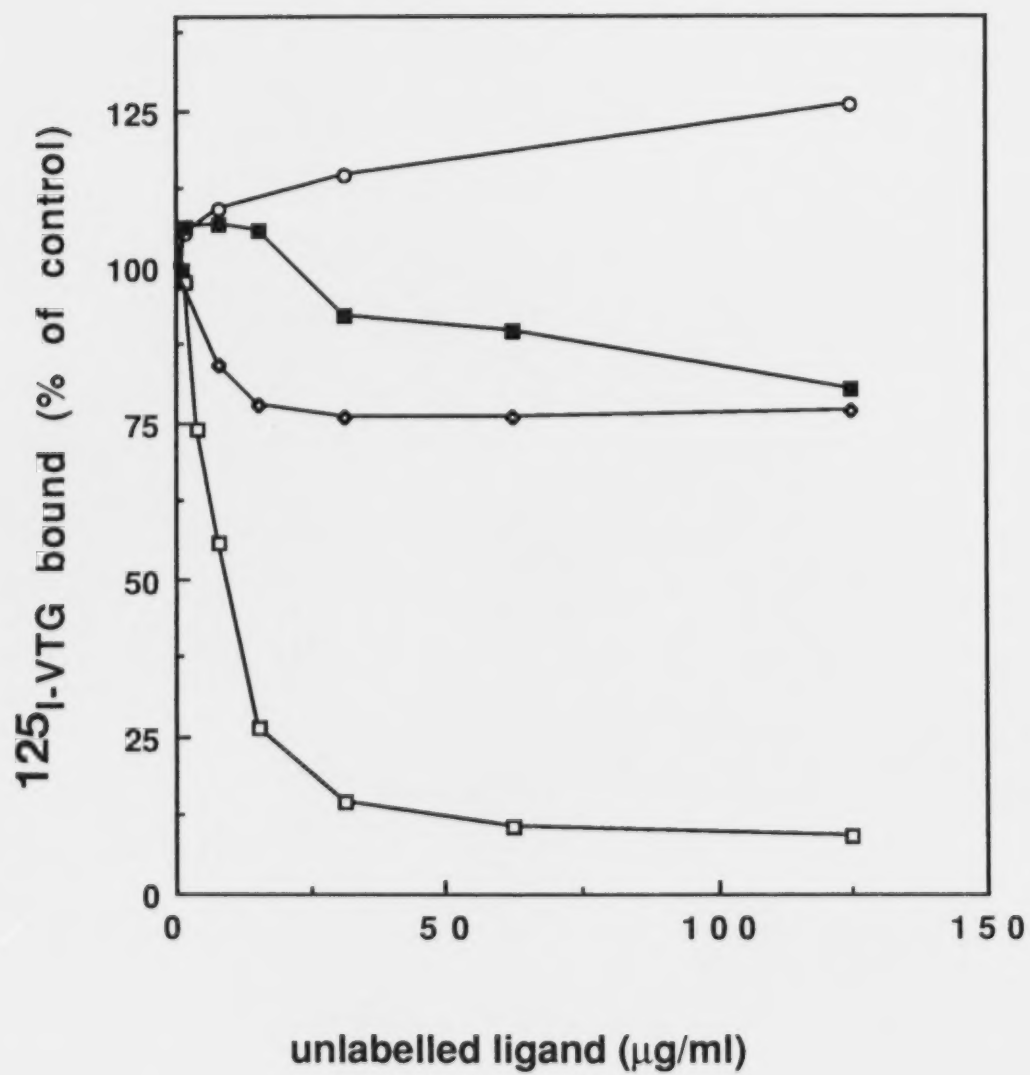


FIGURE IV 16

EFFECT OF UNLABELLED HUMAN APOLIPOPROTEIN E ON THE BINDING OF CHICKEN ^{125}I -VLDL TO THE PURIFIED 95 kDa RECEPTOR

Each assay tube contained the standard assay mixture (100 μl) with 6.7 μg of precipitated immunoaffinity purified receptor and 10 $\mu\text{g}/\text{ml}$ laying hen ^{125}I -VLDL in the presence of unlabelled VLDL (■), reconstituted human apo E3/3 particles (○), or rooster HDL (◆) at the indicated concentrations. The amount of receptor-bound radiolabelled ligand was determined by filtration as described in Chapter II.O. The 100%-of-control value was 19 μg VLDL bound/mg protein.

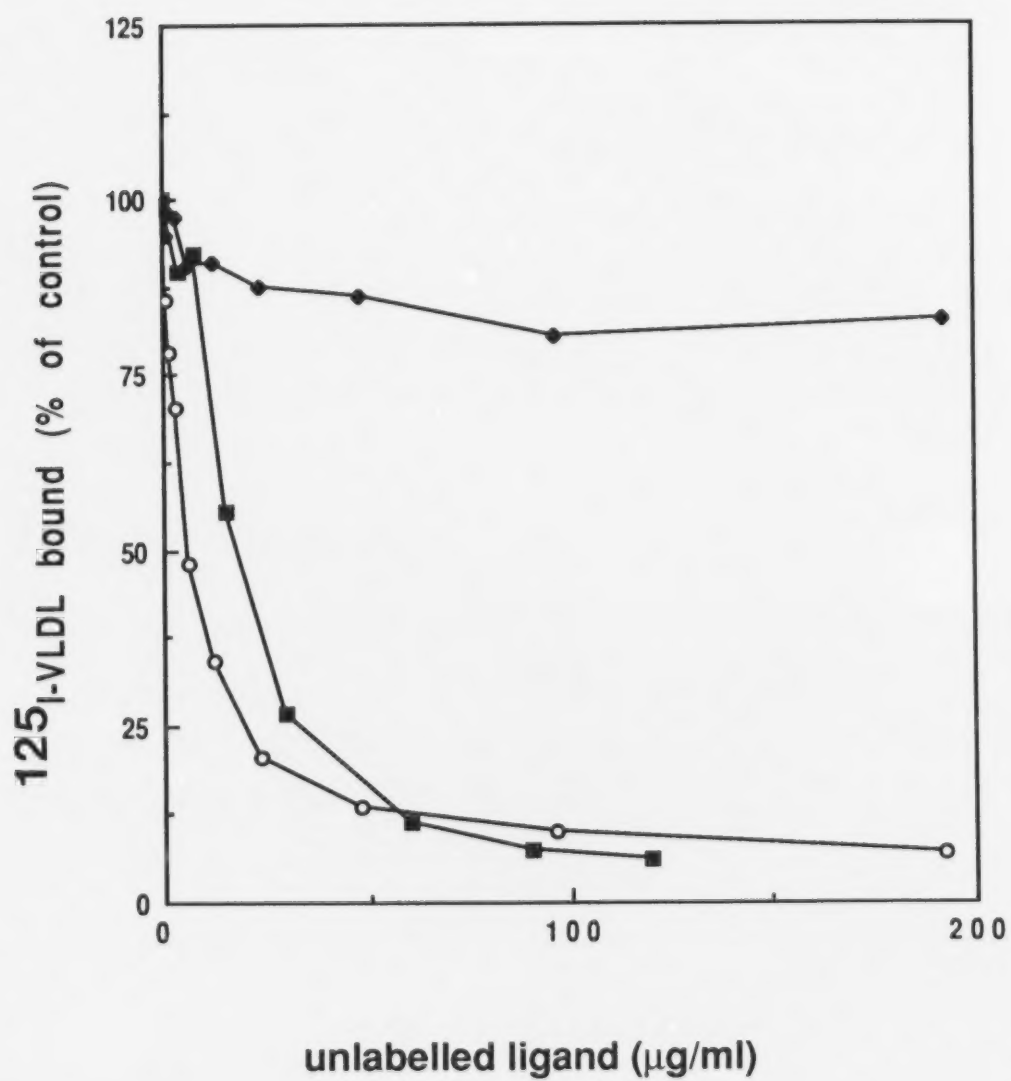
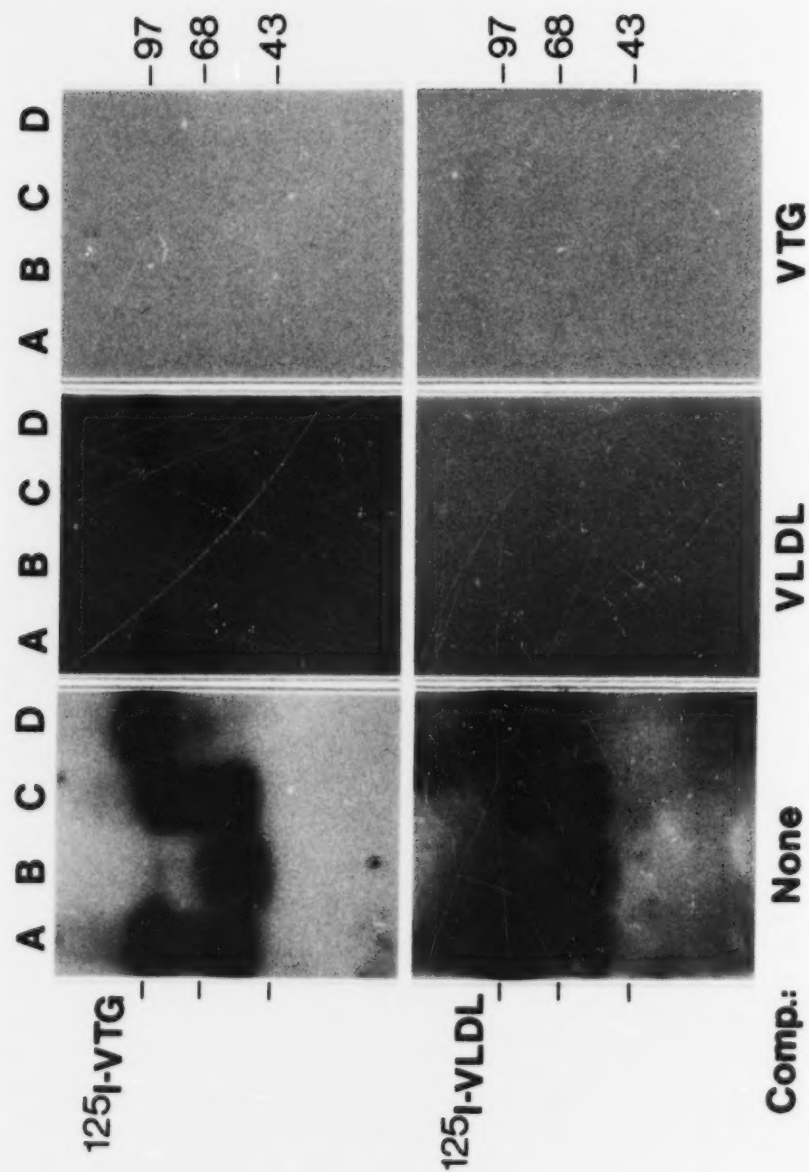


FIGURE IV 17

PROTEASE DIGESTION AND LIGAND BLOTTING ANALYSIS OF THE PURIFIED 95 kDa RECEPTOR

Immunoaffinity purified receptor (150 μ g) was incubated with 1.5 μ g of endoproteinase arg-C (*lanes A*), endoproteinase lys-C (*lanes B*), endoproteinase glu-C (*lanes C*), or without enzyme (*lanes D*) for 18 hr at 37 °C in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl₂, 1 mM PMSF and 30 mM octylglucoside. Equal aliquots were separated by 4.5-18% SDS-polyacrylamide gel electrophoresis in the absence of reducing agents and transferred to nitrocellulose. Replicas were incubated in the presence of ¹²⁵I-VTG (5 μ g/ml, 264 cpm/ng) (upper panels) or ¹²⁵I-VLDL (5 μ g/ml; 36 cpm/ng) (lower panels). A 100-fold excess of unlabelled VLDL (center) or unlabelled VTG (right panels) was added to the incubations as indicated. Strips were exposed to Kodak AR film for 4 hr (¹²⁵I-VTG) or 8 hr (¹²⁵I-VLDL) at -70 °C, respectively.



CHAPTER V

IDENTIFICATION OF THE RECEPTOR BINDING DOMAINS OF AVIAN APOLIPOPROTEIN B

V. A. INTRODUCTION

Site specific mutagenesis of the cysteine-rich ligand binding domain of the LDL receptor has illustrated that negatively charged glutamate and aspartate residues found in SDE repeats were essential for the binding to either apo B or apo E [150], [152]. This suggests that at least in part, the ligand-receptor interaction is ionic in nature. Lysine residues have been shown to be vital for either apo B or apo E-mediated binding to the LDL receptor [134].

The receptor binding domain of apo E has been identified from tryptic and chemical digestion studies and localized to a region spanning residues 140-160 containing a large proportion of positively charged residues. Similar strategies have been employed to identify the receptor binding domains of human apo B. The ability of 40 individual monoclonal antibodies directed against apo B to each inhibit ^{125}I -LDL binding to the LDL receptor was tested. Those antibodies which displayed total inhibition have epitopes located in the T2/T3 hinge region corresponding to a thrombin cleavage site [115].

Receptor mediated binding of avian VLDL to the 95 kDa receptor is facilitated by apo B [201]. Sequence analysis of the carboxy terminal portion of chicken apo B has illustrated 31% nucleotide identity when compared to analogous regions of human apo B. This compares with 50% nucleotide identity between chicken and human apo A I, a 1.0 kb cDNA in both species [246]. Apolipoprotein genes may not be highly conserved between avian and mammalian species.

The uptake of VLDL into the oocyte results in partial proteolysis of apo B [201] by a cathepsin D activity present in the oocyte [247]. Plasma or yolk VLDL displays similar binding properties, indicating that the cathepsin D-mediated proteolysis does not destroy native receptor binding sites. We were interested in taking advantage of this physiological proteolysis to study receptor binding sites in avian apo B. The experiments presented in

this chapter describe localization of the receptor binding sequence of chicken apo B via a new technique termed reverse ligand blotting.

V.B RESULTS

V. B. 1. CHARACTERIZATION OF YOLK VLDL

Initial studies completed by Dr. Nimpf in the laboratory have shown that the delipidated VLDL fraction isolated from egg yolk contained a number of fragments when analyzed by SDS-polyacrylamide gel electrophoresis. In Figure V 1, intact apo B migrating at > 500 kDa is observed in *lane B* isolated from laying hen VLDL. Yolk VLDL is shown in *lane C*; a number of bands are present ranging from 178 to 56 kDa. These have been termed yolk (y) B1-B4, respectively. Note that no intact apo B is present and that apo VLDL-II is unaffected by the processing that occurs upon oocyte uptake. A polyclonal antibody raised against an electroeluted preparation of apo B recognizes all of the bands present in *lane C* plus an additional weakly stained component, termed yB5 migrating at 43 kDa (*lane D*). Polyclonal antibodies were produced against individual fragments yB2, yB3, and yB4. Repeated attempts to produce an antibody directed against yB1 were unsuccessful.

V. B. 2 REVERSE LIGAND BLOTTING

This technique had been developed in the laboratory by Stefano Stifani. He was interested in characterizing the individual lipovitellin proteins that mediated receptor binding. He incubated lipovitellin and phosvitin nitrocellulose replicas with detergent solubilized oocyte extract, followed by an antibody directed against the 95 kDa receptor and finally with ^{125}I -protein A. He observed that the 95 kDa receptor interacted with lipovitellins, but not phosvitin [58].

Since pure 95 kDa receptor was available, a more streamlined protocol was developed to probe the interaction between yolk apo B fragments and the receptor protein. The receptor was radiolabelled with Na^{125}I and then used to probe nitrocellulose replicas containing yolk VLDL. As illustrated in Figure V 1, *lane E*, the ^{125}I -receptor recognizes

three bands present in yolk VLDL, namely, yB1, yB2 and yB4. The addition of 10 mg/ml suramin to the incubation mixture abolishes this interaction (*lane F*).

Reverse ligand blotting was completed on a larger scale as shown in Figure V 2. A Coomassie stain of various plasma and yolk proteins is presented in Panel A. Plasma and yolk VLDL are illustrated as described above in *lanes 1* and *2*. Plasma VTG, migrating at 220 kDa, is observed in *lane 5*. VTG is processed into lipovitellins and phosvitins upon oocyte uptake. The family of proteins termed lipovitellins is shown in *lane 4* migrating at 30, 41, 78 and 110 kDa. Crude yolk was delipidated (*lane 3*) and comparison of this sample with yolk VLDL and lipovitellin illustrates that the majority of yolk proteins are derived from VLDL and VTG.

When a nitrocellulose replica was probed with ^{125}I -receptor, the results in Panel B were observed. The radiolabelled receptor binds to intact VLDL and VTG as shown in *lanes 1* and *5*, respectively. The receptor binds to yB1, yB2 and yB4 (*lane 2*) as shown before and also shows weak interaction with yB3 and yB5. As demonstrated previously, the 95 kDa receptor binds to the 30, 78, and 110 kDa lipovitellin proteins (*lane 4*) [58]. In whole yolk (*lane 3*), the only proteins that demonstrate binding of the radiolabelled receptor are those present in the yolk VLDL and lipovitellin fractions. Suramin inhibits 95 kDa receptor binding to all VLDL and VTG-related proteins, as observed in previous ligand blotting experiments and solid phase filtration assays (panel C).

V. B. 3. SEQUENCE DETERMINATION

Amino terminal sequencing of fragments yB1-yB4 was completed by Mike Carpenter in Dr. Smillie's laboratory. Yolk B1 and yolk B2 had blocked amino termini, but yolk B3 could be aligned with residues 2652-2671 from the human apo B sequence, as shown in Figure V 3. Discrete sequence information was also obtained for yolk B4, however, attempts to align this sequence with human or chicken apo B were unsuccessful.

Sequencing of internal peptides from yB2 and yB3 was conducted in collaboration with Dr. R. Aebersold. Two peptides from yolk B2 aligned with published sequence of

chicken apo B [31] as shown in Figure V 4. The first peptide shows weak homology with human apo B as has been previously illustrated [31]. Three peptides were sequenced from yolk B3. These peptides aligned with human apo B in the same region as shown for the amino terminal sequence of yolk B3. Peptides three and four were approximately 50% identical to human apo B with 11 of 22 and 10 of 18 identical residues, respectively, whereas peptide five was from a highly conserved region with 5 of 6 identical residues.

V. C. DISCUSSION

Previous investigations in the laboratory have illustrated that the proteolytic processing of apo B and VTG that occurs following the endocytic uptake of these ligands does not affect nascent receptor binding sites expressed in the corresponding fragments. The receptor binding domain of apo E has been localized to a single linear region. For apo B, a monoclonal antibody blocks ^{125}I -LDL binding with a stoichiometry of 1:1 (antibody:LDL), which suggests a single discrete binding site is present on this ligand. Thrombin digestions of apo B illustrates that four individual fragments are produced. Five different anti apo B monoclonal antibodies block the interaction between ^{125}I -LDL and the 130 kDa LDL receptor. The epitopes for these antibodies are found in three discrete regions at the T2/T3 junction corresponding to residues 2980-3084 (4G3), 3441-3569 (5E11, 3A10, MB47), and 3665-3780 (B1B3). Although this represents 800 amino acids of linear sequence, it is known that higher secondary structure may exist due to the presence of a disulfide bond between residues 3167 and 3297 [96]. In addition, a patient showing moderate to severe hypercholesterolemia and defective binding of apo B to the LDL receptor was shown to express a R to Q mutation at position 3500, adjacent to the T2/T3 junction [248], [249].

Upon uptake of VLDL into the oocyte, cathepsin D converts apo B into a series of five major fragments. Morphological and biochemical characterization of yolk VLDL

shows no changes in structure or lipid composition upon uptake of VLDL into the oocyte [201].

Ligand blotting is successful for the 95 kDa oocyte VLDL/VTG receptor when it is electrophoresed under non-reducing conditions only. The disulfide structure of this protein is unaffected by SDS and partial renaturation of the protein is thought to occur during electrophoretic transfer to nitrocellulose.

Reverse ligand blotting experiments show that purified, radiolabelled 95 kDa receptor interacts with three yolk apo B fragments: yolk B1 (175 kDa), yolk B2 (79 kDa) and yolk B4 (56 kDa). The yolk VLDL sample had been reduced for this analysis; however, the same pattern of proteins is present when the delipidated sample is resolved by SDS-polyacrylamide gel electrophoresis under non-reducing conditions. YB1, yB2 and yB4 all bound the 95 kDa receptor under non-reducing conditions as verified by immunoblotting (data not shown), indicating that disulfide structure may not be a factor in determining the receptor binding domain of chicken apo B.

The reverse ligand blotting experiment is sensitive to the amount of ligand present on the nitrocellulose. Although receptor interaction with yB3 and yB5 was observed in Figure V 2, binding to these fragments was variable and dependent on the protein concentration. In other experiments, ^{125}I -receptor bound non-specifically to apo VLDL-II in reverse ligand blots if the protein loaded per well exceeds 40 μg (data not shown). The buffer utilized for reverse ligand blotting experiments was the same buffer used for ligand blotting with the addition of 150 mM NaCl and 0.2% (v/v) Triton X-100. The presence of the detergent appeared to lower the background binding.

Reverse ligand blotting is a useful strategy for identifying receptor binding fragments found in yolk VLDL. From the experiments presented in this chapter it is evident that the receptor binding domain of apo B has been minimally localized to 56 and 79 kDa yolk apo B fragments. Further proteolytic digestion could potentially isolate a smaller domain.

Chicken yolk B2, a 79 kDa yolk apo B fragment, resides at the carboxy terminus of chicken apo B as determined from amino acid sequencing. This apo B fragment binds the 95 kDa receptor in reverse ligand blotting experiments. Human apo B-48 is unable to bind to the human LDL receptor implying that the receptor binding domain of apo B is found in the carboxy terminal portion of apo B. The receptor binding domain of human apo B is proposed to be located at the T2/T3 hinge region stretching over 800 amino acids of apo B-100 as localized from monoclonal antibody mapping. Although yolk B2 is found outside of this region it does reside in the carboxy terminal portion of apo B. Yolk B3 was localized to a region of human apo B starting at residue 2702. Three other internal peptides were sequenced and their alignment with human apo B confirmed the location of yolk B3 within the apo B sequence. Further experiments to obtain the amino terminal sequences of yB2 and yB4 will be completed. It will be important to determine if all yolk apo B fragments represent unique linear sequence or have arisen through partial cathepsin D digestion.

FIGURE V 1

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND REVERSE LIGAND BLOTTING OF YOLK VLDL

All samples were analyzed on a 4.5-18% SDS-polyacrylamide gradient gel in the presence of 50 mM dithiothreitol. *Lane A*, M_r standards, (myosin, 200 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.3 kDa); *lane B*, 5 μ g of plasma VLDL; and *lanes C-F*, 15 μ g of yolk VLDL. Proteins in *lanes B* and *C* were stained with Coomassie blue, while proteins in *lanes D-F* were electrophoretically transferred to nitrocellulose. *Lane D* was incubated with 25 μ g/ml of anti-chicken apo B IgG followed by 1.7 μ g/ml of ^{125} I-Protein A (1200 cpm/ng). *Lanes E* and *F*, 5.0 μ g/ml ^{125} I-labelled 95 kDa receptor (28 cpm/ng) in the presence (*F*) or absence (*E*) of 10 mg/ml suramin. Exposure of Kodak AR film was for 10 hr (*lane D*) or 24 hr (*lanes E* and *F*) at -70°C .

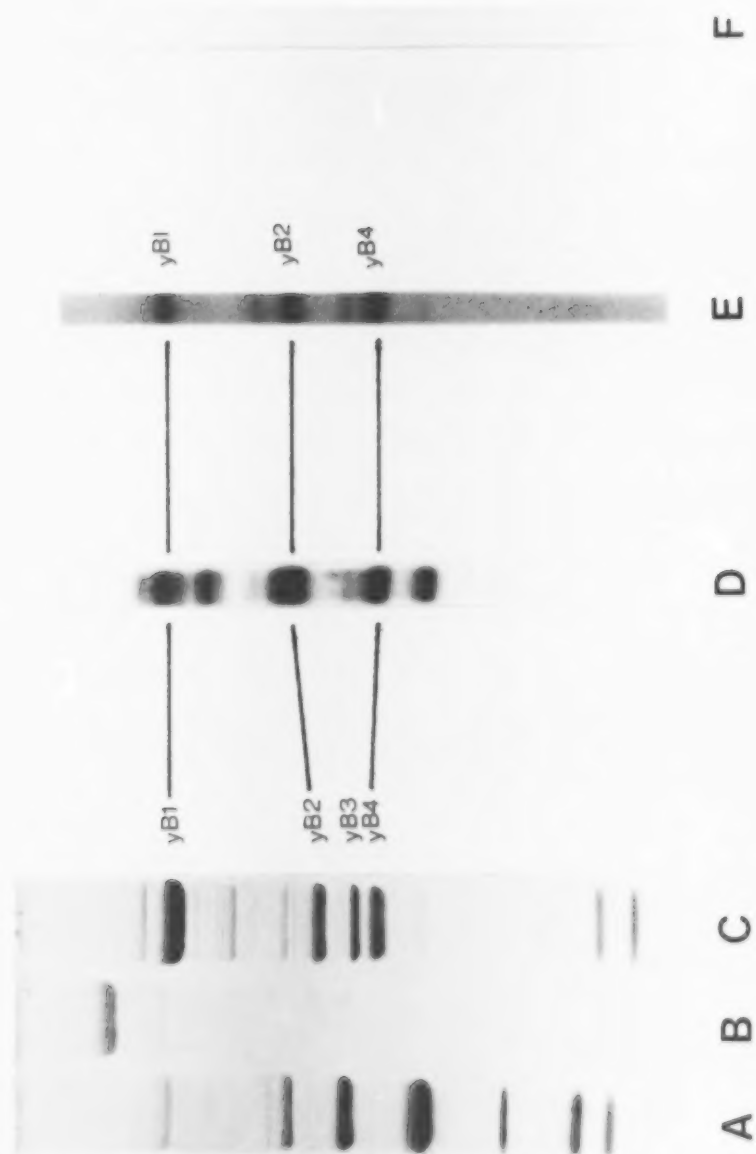


FIGURE V 2

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND REVERSE LIGAND BLOTTING OF PLASMA AND YOLK PROTEINS

All samples were analyzed on a 4.5-18% SDS-polyacrylamide gradient gel in the presence of 50 mM dithiothreitol. The M_r standards (myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 29 kDa) are shown. Panel A: *lane 1*, 5 μ g of plasma VLDL; *lane 2*, 25 μ g of yolk VLDL; *lane 3*, 60 μ g of whole yolk; *lane 4*, 20 μ g of lipovitellin; *lane 5*, 5 μ g of VTG. The proteins were stained with Coomassie blue. Panels B and C: each lane contained the fractions indicated in panel A; following electrophoretic transfer to nitrocellulose, the strips were incubated with 5.0 μ g/ml of ^{125} I-labelled 95 kDa receptor (28 cpm/ng) in the presence (C) or absence (B) of 10 mg/ml suramin. Exposure of Kodak AR film was for 89 hr at -70°C . M_r standards are indicated.

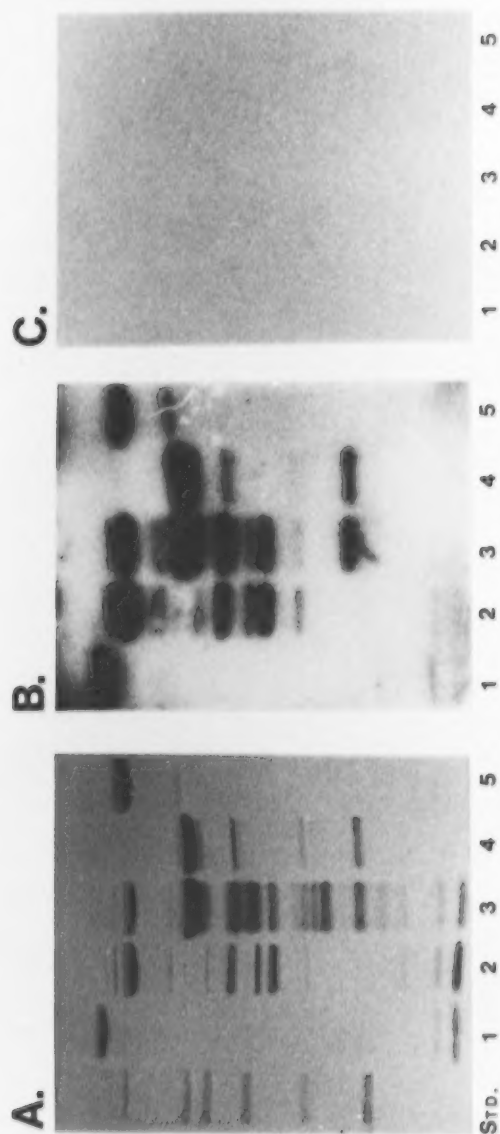


FIGURE V 3

SEQUENCE ALIGNMENT OF HUMAN APO B WITH CHICKEN YOLK B3

The amino acid sequence of yolk B3 is aligned with the homologous sequence in human apo B [96]. Numbers refer to the position of the amino terminal residue in human apo B [96]. Identical residues appear in bold face. In the consensus sequence "Ø" refers to a hydrophobic residue, " - " to a negatively charged amino acid and " + " to a positively charged amino acid.

Human apo B2702	NDFQVPDLHIPEFQLPHISH
Chicken yolk B3	NEFQIPDLKTPEFQLPHISH
Consensus	N - FQØPDL + ØPEFQLPHISH

FIGURE V 4

SEQUENCE ALIGNMENT OF HUMAN APO B WITH TRYPTIC PEPTIDES FROM CHICKEN YOLK B2 AND YOLK B3

The amino acid sequence of tryptic peptides of yolk B2 and yolk B3 is aligned with the sequences of chicken apo B (corresponding to yolk B2) [31] and human apo B [96] derived from cDNA cloning. Numbers refer to the position of the amino terminal residue in human apo B. "X" denotes an unidentified residue. Identical residues appear in bold face.

Human apo B4226 L P F E L R K H K

Chicken yolk B2 V P A S E T I L R

Chicken apo B (cDNA) V P A S E T I L R

Human apo B4472 L Q D F S D Q L S D Y Y E

Chicken yolk B2 X X E I Y G Q L S D S Q D

Chicken apo B (cDNA) L Q E I Y G Q L S D S Q E

Human apo B2737 I Q S P L F T L D A N A D I G N G T T S A N

Chicken yolk B3 X Z S P F F T L S T Q A E V G V T T A S A N

Human apo B3001 V I G T L K N S L F F S A Q P F E I

Chicken yolk B3 X T G T V N N D F F F L V Q P F E I

Human apo B3066 F N Q Y K Y

Chicken yolk B3 F N Q Y V Y

CHAPTER VI

THE OOCYTE EXPRESSES A 380 kDa LDL RECEPTOR-RELATED PROTEIN³

³ Reprinted, with permission, Stifani, S., Barber, D. L., Aebersold, R., Steyrer, E., Shen, X., Nimpf, J. and Schneider, W. J. *J. Biol. Chem.* **266**: 19079-19087, 1991. Copyright, 1991, by The American Society for Biochemistry and Molecular Biology, Inc.

VI.A. INTRODUCTION

Studies of familial hypercholesterolemia by Brown and Goldstein [141] led to the description of the LDL receptor gene in 1985. This protein has been the subject of extensive investigation and today it is known that the LDL receptor functions to transport apo B-containing lipoproteins from the circulation into peripheral tissues. Its various domains have been correlated with function through the identification of FH patients harbouring various mutations.

In recent years, new members of the LDL receptor family have been reported. In 1987, partial sequence information from glycoprotein (gp) 330, the autoantigen in Heymann nephritis, was described [176]. Sequence information derived from six discrete regions reveal 13 cys-rich type A ligand binding repeats as well as 3 NPXY sequences. Immunolocalization studies have shown gp 330 to be localized to coated pits. This implies that gp 330 may be involved in an endocytic pathway, but the physiological function of this protein is unknown, as is its ability to bind lipoproteins.

During screening of cDNA libraries in search of sequences that hybridize with complementary probes, a 15 kb cDNA resembling the LDL receptor was isolated. The so-called LDL receptor-related protein (LRP) contains 31 type A repeats and 23 type B repeats organized in 4 clusters, which appear to have arisen from an exon cassette duplication [166]. No O-linked carbohydrate region is present; however, LRP is endowed with two NPXY internalization sequences, indicating that it could partake in an endocytic pathway. Recent studies have shown that the 600 kDa LRP precursor is processed at a unique sequence, RHRR, into LRP-515 and LRP-85 [167]. The functional properties of this protein are complex. There is some evidence to suggest that LRP binds chylomicron remnants which are thought to be targeted to sites of catabolism via apo E. Recombinant apo E enriched β -VLDL particles, isolated from cholesterol fed rabbits, bind to LRP [168].

The α_2 -macroglobulin receptor has been shown to be identical to LRP, indicating that LRP could act as a multifunctional transporter [174], [250].

In avian species, two members of the LDL receptor have been described. A single 95 kDa oocyte membrane protein mediates the transport of VLDL [224], [251] and VTG [64], [230] into the oocyte. In addition, apo E binds to the 95 kDa receptor [203]. This is an interesting finding as apo E is not synthesized in birds, whereas VTG is not found in mammalian species. Two predominantly basic sequences within VTG align well with the putative apo E receptor binding sequence. Cholesterol homeostasis in somatic tissues of the laying hen is maintained by the 130 kDa LDL receptor. Interestingly, the mammalian LDL receptor is immunologically related to the 95 kDa chicken oocyte receptor, but not to the avian 130 kDa LDL receptor.

Stefano Stifani has demonstrated that a putative chicken LRP is expressed in somatic tissues [252]. This protein appears as a 515 kDa processed form in ligand blots with ^{125} I-VTG, immunoblots with anti-chicken LRP, anti-rat LRP antibodies, and Ca^{2+} overlays. Experiments in this chapter will provide evidence that a 380 kDa protein is found in oocyte membranes, representing the oocytic counterpart to somatic LRP-515. This protein was purified and its ligand binding properties were analyzed. In addition, amino acid sequence information illustrates a similarity with mammalian LRP and the protein was shown to be co-localized with the 95 kDa VLDL/VTG receptor in immunocytochemistry experiments.

VI. B. RESULTS

VI. B. 1. ALKYLATION OF DETERGENT SOLUBLIZED OOCYTE MEMBRANE PROTEINS

A number of observations suggested the presence of another VLDL binding protein in oocyte membranes in addition to the previously described 95 kDa VLDL/VTG receptor. During purification of the 95 kDa protein, VLDL-Sepharose chromatography enriched a higher M_r protein migrating at 380 kDa, in addition to the VLDL receptor (see Figure IV

2). This protein bound ^{125}I -VLDL in ligand blots, but was distinct from receptor dimer as shown by immunoblotting. To further verify this result, alkylation of oocytes was conducted, prior to homogenization and solubilization. Both iodoacetic acid and N-ethylmaleimide were tested as alkylating reagents; a 10 mM concentration of either compound was added to all buffers throughout membrane preparation and solubilization. Figure VI 1 illustrates that the mobility of the 380 kDa protein ($\diamond$) with prior alkylation using iodoacetic acid (*lane F*) or N-ethylmaleimide (*lane G*) was altered (*lanes C and D*). Indeed, the mobility of this band decreased ($\triangleright$), indicating that the 380 kDa protein also has a high disulfide content. The newly described protein bound ^{125}I -VLDL in ligand blotting experiments (*lanes H-J*), even if the sample had been alkylated, indicating that no free cysteine groups are involved in ligand binding. Binding of ^{125}I -VLDL to the 380 kDa protein and the 95 kDa receptor ($\blacklozenge$) was abolished when the samples were reduced prior to electrophoresis as shown in *lanes K-M*.

VI.B.2. TRITON X-114 PHASE SEPARATION OF OOCYTE AND LIVER MEMBRANE PROTEINS

Triton X-114 has been useful for the characterization of membrane proteins, as it undergoes a phase separation at temperatures above 23 °C, the cloud point for this detergent [208]. An aqueous phase is detergent-poor and peripheral membrane proteins are found localized to this layer, whereas the detergent phase contains Triton X-114 at concentrations exceeding the CMC; integral membrane proteins are concentrated in this phase. Triton X-114 was used to solubilize liver and oocyte membrane proteins and then phase separations were carried out, by incubating extract at 37 °C for 10 min, followed by centrifugation to separate the phases. Both phases were washed extensively in order to yield distinct preparations. A Coomassie stain of Triton X-114 crude extract as well as aqueous and detergent phases of oocyte (*lanes 1-3*) and liver membranes (*lanes 4-6*) is shown in Figure VI 2, Panel A. In both tissues, peripheral membrane proteins predominate as few proteins are found in the detergent phase. Ligand blotting experiments

with ^{125}I -VTG show that two VTG binding proteins are observed in Triton X-114 solubilized oocyte membranes (Panel B, *lane 1*). One protein migrating at 380 kDa ($\diamond$) is localized to the aqueous phase (*lane 2*), whereas the 95 kDa receptor ($\blacklozenge$) is found primarily in the detergent phase (*lane 3*). Liver membrane preparations contain a high M_r ^{125}I -VTG binding protein (Panel B, *lane 4*) ($\star$) that is localized in the aqueous phase (*lane 5*). This represents a processed form, LRP-515 as demonstrated by Stefano Stifani [252]. Overlays using $^{45}\text{Ca}^{2+}$ have been shown to be useful in identifying LRP and the LDL receptor from rat tissues [215]. Ca^{2+} blots decorate the same proteins as identified with ^{125}I -VTG ligand blotting (panel C): a liver protein of ~ 500 kDa found in the aqueous phase (*lanes 4* and *5*), a 380 kDa oocyte peripheral membrane protein (*lanes 1* and *2*) and a 95 kDa integral membrane protein (*lanes 1* and *3*). The aqueous phase contains small amounts of the 95 kDa receptor. This may indicate that this protein undergoes a specific interaction with the 380 kDa protein or other peripheral membrane proteins. The remaining experiments will provide evidence that the 380 kDa protein is the oocyte LRP.

VI.B.3. PURIFICATION OF THE 380 kDa OOCYTE MEMBRANE PROTEIN

Oocyte membrane proteins were detergent solubilized using Triton X-100. This material was diluted five-fold with buffer and applied to an immunoaffinity column consisting of an anti-oocyte LRP antibody covalently coupled to CNBr-activated Sepharose 4B. This antibody was obtained by injection of the 380 kDa protein eluted from a SDS-polyacrylamide gel. The sample applied to the gel had been enriched in both the 380 kDa protein and the 95 kDa receptor by VLDL-Sepharose 4B chromatography (see Figure IV 2). The diluted oocyte membrane extract was allowed to recycle over the column and then the column was washed with a buffer containing 1 M NaCl. Bound protein was eluted in the presence of 0.5 M NH_4OH . Purified 380 kDa protein was obtained from this single rapid chromatography step as shown in Figure VI 3, *lane C*. The mobility of this protein can be compared to apo B-100 (*lane A*), which migrates at 512 kDa.

VI. B. 4. LIGAND BLOTTING

Ligand blotting of partially purified LRP was conducted. ^{125}I -VLDL and ^{125}I -VTG interact with two proteins of 95 and 380 kDa (Figure VI 4, lanes A and D). In this preparation, the oocyte LRP has been enriched (compare with Figure VI 1) but some 95 kDa receptor is still present. Both a 100-fold excess of unlabelled VLDL (lane B) or unlabelled VTG (lane C) abolished ^{125}I -VLDL binding to the 95 kDa receptor and the oocyte LRP. However, only unlabelled VTG could compete for ^{125}I -VTG binding (lane D). A 100-fold excess of unlabelled VLDL (lane E) did not compete for ^{125}I -VTG binding to oocyte LRP and resulted in partial competition for binding of ^{125}I -VTG to the 95 kDa receptor as observed previously (Figures IV 4 and 17).

VI. B. 5. SEQUENCE DETERMINATION

Partially purified oocyte LRP (VLDL-Sepharose 4B eluate) was transferred to nitrocellulose. The area of the nitrocellulose sheet corresponding to the 380 kDa protein as determined by Ponceau S staining was treated with trypsin and the released peptides were sequenced by Dr. R. Aebersold. Three of these peptides could be aligned with the derived human LRP cDNA sequence. The first peptide was highly similar to two regions found within the YWTD repeats starting at residues 372 and 1420. A peptide with 18 identified residues aligned with a repeat structure found within human LRP as shown in Figure VI 5. The chicken sequence conformed in 13 out of 15 positions to the derived consensus sequence, while the optimally aligning sequence found in the human cognate matched at 12 positions. The sequence aligning closest to the carboxy terminus of human LRP, starting at residue 3975, lies within the membrane anchored light chain of LRP which is one of two subunits arising by post-translational proteolytic processing from a 4525 residue single chain precursor [167]. Finally, the third peptide aligns with a region found in the cytoplasmic domain of human LRP between the two putative internalization signals [165], NPTY (residues 4451-4454) and NPVY (residues 4485-4488). The amino acid sequence

analysis of tryptic fragments of the 380 kDa oocyte membrane protein indeed provide strong support for its identity with a chicken homologue of mammalian LRP.

VI. B. 6. QUANTITATIVE BINDING ANALYSIS

The ligand binding properties of the putative chicken oocyte LRP are illustrated in Figures VI 6-8. This protein binds VLDL, LDL and VTG with high affinity, in a saturable and specific fashion. Scatchard analysis [223] reveals a single class of binding sites for VLDL and LDL with K_d values of 2.60 $\mu\text{g/ml}$ (2.7 nM) for VLDL and 1.59 $\mu\text{g/ml}$ (3.1 nM) for LDL. However, graphical representation of the VTG binding data reveals two classes of binding sites, a low affinity site with a K_d value of 3.86 $\mu\text{g/ml}$ (8.7 nM) and a high affinity site with a K_d of 56.8 $\mu\text{g/ml}$ (129 nM). B_{max} values were not reported due to the loss of receptor ligand complexes associated with this assay, as discussed earlier for experiments with the 95 kDa receptor (see Chapter IV. B. 4).

VI. B. 7. COMPETITIVE BINDING

In order to test the specificity of ligand binding, competition experiments were conducted with oocyte LRP and sub-saturating concentrations of radiolabelled ligand in the presence of increasing concentrations of unlabelled ligands as described in Chapter IV. Only unlabelled VLDL competes for ^{125}I -VLDL binding to the 380 kDa protein, as shown in Figure VI 9. Excess concentrations of unlabelled HDL are unable to compete for binding. Unlabelled VTG shows slight enhancement of ^{125}I -VLDL binding, which probably can be attributed to mixed ligand artifacts arising from the assay. Reductively methylated chicken VLDL competes for 35% of ^{125}I -VLDL binding at 50 $\mu\text{g/ml}$. The reductive methylation of lysine residues in this preparation may not have been complete.

Like the 95 kDa receptor (Figure IV 13), binding of ^{125}I -VTG to chicken oocyte LRP can only be competed by unlabelled chicken VTG as observed in Figure VI 10. Unlabelled VLDL is unable to compete for binding. Lysine residues must also be important for the VTG interaction with chicken oocyte LRP, as reductively methylated

VTG shows no competition for ^{125}I -VTG binding. Chicken HDL shows partial competition, but at no time does this exceed 20% of total binding.

VI. B. 8. IMMUNOCYTOCHEMISTRY

The biochemical experiments described in Chapters IV and VI provide convincing evidence that two distinct proteins bind the major yolk precursors, VLDL and VTG. In order to obtain further information on these proteins, an immunocytochemical investigation was conducted. The 95 kDa receptor and the 380 kDa oocyte LRP were co-localized to vesicular structures from within a 2 mm oocyte (Figure VI 11). It was not possible to perform negative staining of this sample because of the experimental conditions employed to visualize the gold particles, but it is assumed that these vesicles are coated. Dr. Shen has isolated oocyte coated vesicles in the laboratory and has shown that the 95 kDa receptor and oocyte LRP are both found in this fraction [253]. This indicates that these two proteins may play a co-operative role in receptor mediated transport of VLDL and VTG into the oocyte.

VI. C. DISCUSSION

The major co-purifying protein during purification studies of the oocyte 95 kDa VLDL/VTG receptor is a 380 kDa protein, that shared many properties with the smaller receptor. Specifically, the 380 kDa protein binds the identical ligands, namely, VLDL, LDL, and VTG, as well as displaying similar biochemical properties during purification. This protein co-elutes with the 95 kDa receptor from VLDL-Sepharose 4B and DEAE-52 Cellulose. The 380 kDa protein appears to be the processed oocyte equivalent of the somatic LRP-515, as shown by similar binding properties as well as identical distribution in the aqueous phase of Triton X-114 solubilization experiments of each putative, processed LRP.

Dimerization of the mammalian LDL receptor readily occurs and has been shown to be mediated via cys 807 in the cytoplasmic domain [225]. It was initially believed that the low migrating co-purifying protein isolated during VLDL-Sepharose 4B chromatography

was a 95 kDa receptor dimer, as it displayed binding of ^{125}I -VLDL in ligand blots. However, on closer inspection this protein migrates at a position much greater than 200 kDa. Figure IV 3 clearly illustrates that the high M_r protein isolated from VLDL-Sepharose 4B chromatography is distinct from receptor dimer which is found in purified preparations of the 95 kDa receptor. Alkylation experiments presented in this chapter confirm this observation. The 380 kDa protein is present when oocyte membranes have been alkylated prior to homogenization and solubilization with Triton X-100 and resolved by SDS-polyacrylamide gel electrophoresis in the presence of 50 mM DTT, thereby illustrating that it is a monomeric protein.

Triton X-114 has been useful to distinguish between peripheral and integral membrane proteins. Cleavage of intact LRP-600 is known to occur at a basic RHRR sequence [167] to generate LRP-515 and LRP-85 in mammalian species. LRP-515 contains the majority of the type A and B repeats whereas a small number of type B repeats, the transmembrane region and the cytoplasmic domain are found in LRP-85. Phase separations using Triton X-114 illustrated that the high M_r ^{125}I -VTG- and $^{45}\text{Ca}^{2+}$ - binding proteins both partitioned to the aqueous phase. The only proteins that interact with $^{45}\text{Ca}^{2+}$ are members of the LDL receptor family. The liver LRP corresponds to LRP-515 originally described in mammalian species, however, the oocyte protein has a M_r of 380 kDa.

The identity of the 380 kDa protein as a member of the LRP family was confirmed by sequence analysis of tryptic peptides. Three distinct sequences were shown to align with unique sequence derived from cDNA cloning of human LRP. These three sequences correspond to signature sequences characteristic of human LRP [166]. A high degree of sequence similarity was observed for one sequence indicating that LRP genes have undergone conservation throughout evolution of avian and mammalian species.

The purified oocyte LRP-380 binds VLDL, LDL and VTG with high affinity, in a specific and saturable fashion. A single class of binding sites is observed for the binding

of VLDL and LDL whereas high and low affinity binding sites are observed for VTG. This is not surprising when the size of LRP-380 and potential for multivalency are considered. Somatic LRP may also act as the receptor for α_2 -macroglobulin, a protease trap. When the ligand is activated through proteolytic action, the α_2 -macroglobulin-proteinase complexes are removed from the circulation by the receptor. α_2 -macroglobulin can be activated *in vitro* by treatment with methylamine. Dr. Shen in the laboratory has shown that activated avian α_2 -macroglobulin binds to both the oocyte LRP and 95 kDa VLDL/VTG receptor [252]. Solid phase binding experiments with activated avian α_2 -macroglobulin have not been tested to date.

Either unlabelled VLDL or unlabelled VTG compete for ^{125}I -VLDL binding to the 95 kDa receptor, whereas only unlabelled VTG was able to displace specific ^{125}I -VTG binding. Discrete ligand binding sites for VLDL and VTG exist on oocyte LRP-380, as specific binding was abolished in the presence of the same unlabelled ligand only, for both VLDL or VTG. Competition experiments with activated α_2 -macroglobulin have not been completed.

Attempts to culture oocytes have been unsuccessful, as a result, knowledge of the itinerary of the 95 kDa receptor of LRP-380 is unknown. The 95 kDa receptor may recycle, analogous to its 130 kDa somatic counterpart. It is difficult to speculate that LRP-380 may recycle as this protein does not contain a transmembrane region. However, it is known that LRP-515 still associates with LRP-85 after processing in mammalian species [167].

The experiments presented within this chapter provide evidence that two discrete LRPs are synthesized in chickens. Somatic tissues synthesize an intact LRP-600 that is post-translationally cleaved into LRP-515 and LRP-85 as has been previously described [167]. Oocyte tissues have a LRP-380 protein which is a processed form of a larger, as yet undescribed precursor. A monoclonal antibody directed against the carboxy terminus of human LRP fails to react with any proteins in somatic or follicle membrane preparations. However, a polyclonal anti-peptide antibody raised against a sequence derived from cDNA

cloning of the chicken somatic LRP recognizes two proteins from chicken somatic tissues: intact LRP-600 and the processed LRP-85 chain. The processed oocyte LRP-85 remains to be identified.

FIGURE VI 1

ALKYLATION AND REDUCTION OF DETERGENT SOLUBILIZED OOCYTE MEMBRANE PROTEINS

All samples were analyzed on a 4.5-18% SDS-polyacrylamide gradient gel; *lanes A-D*, and *K-M* were separated in the presence, and all other samples in the absence of 50 mM dithiothreitol. *Lane A*, M_r standards, (myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; and ovalbumin, 43 kDa); *lanes B, E, H, and K*, 35 μ g of Triton X-100 solubilized oocyte membrane extract from normal oocyte follicles; *lanes C, F, I, and L*, 35 μ g of Triton X-100 solubilized oocyte membrane extract from follicles that have been incubated in the presence of 10 mM iodoacetamide; *lanes D, G, J, M*, 35 μ g of Triton X-100 solubilized oocyte membrane extract from follicles that have been incubated in the presence of 10 mM N-ethylmaleimide. Panel I was stained with Coomassie blue. Following electrophoretic transfer to nitrocellulose, the strips in panel II were incubated with 2.4 μ g/ml ^{125} I-VLDL (117 cpm/ng) as described in "Materials and Methods". Exposure of Kodak AR film was for 18 hr at -70°C . The migration of the 95 kDa receptor ($\blacklozenge$), and the 380 kDa protein in the presence ($\blacktriangleright$) and absence of dithiothreitol ($\diamond$) are indicated.

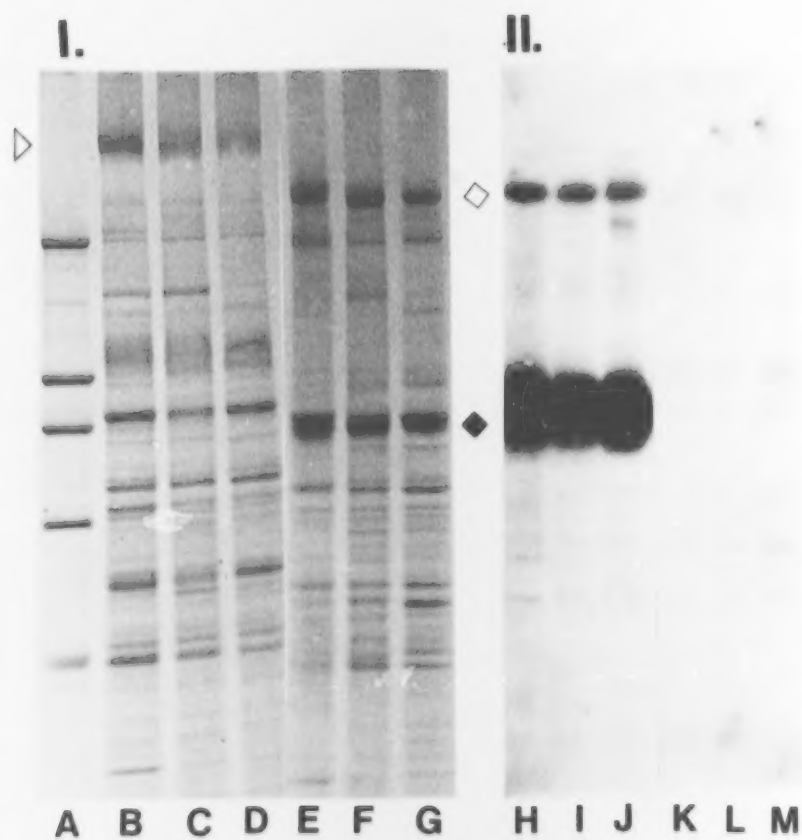


FIGURE VI 2

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND LIGAND BLOTTING OF CHICKEN OOCYTE MEMBRANE TRITON X-114 EXTRACTS

All samples were analyzed on a 4.5-18% SDS-polyacrylamide gradient gel. The M_r standards (myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 29 kDa) were separated in the presence, and all other samples in the absence of 50 mM dithiothreitol. Panel A: *lane 1*, 20 μ g of Triton X-114 oocyte membrane detergent extract; *lane 2*, 75 μ l of Triton X-114 solubilized oocyte membrane aqueous phase; and *lane 3*, 75 μ l of Triton X-114 solubilized oocyte membrane detergent phase; *lane 4*, 35 μ g of Triton X-114 detergent solubilized liver membrane extract; *lane 5*, 75 μ l of Triton X-114 solubilized laying hen liver membrane aqueous phase; and *lane 6*, 75 μ l of Triton X-114 solubilized laying hen liver membrane detergent phase. The proteins were stained with Coomassie blue. Panels B and C: following electrophoretic transfer to nitrocellulose, the strips were incubated with 3.6 μ g/ml ^{125} I-VTG (478 cpm/ng) (B), or ^{45} CaCl₂ (1 μ Ci/ml) (C) as described in "Materials and Methods". Exposure of Kodak AR film was for 4 hr (panel B) or 48 hr (panel C) at -70 °C. The mobility of the 95 kDa receptor (◆), oocyte LRP-380 (◇) and liver LRP-515 (☆) are indicated.

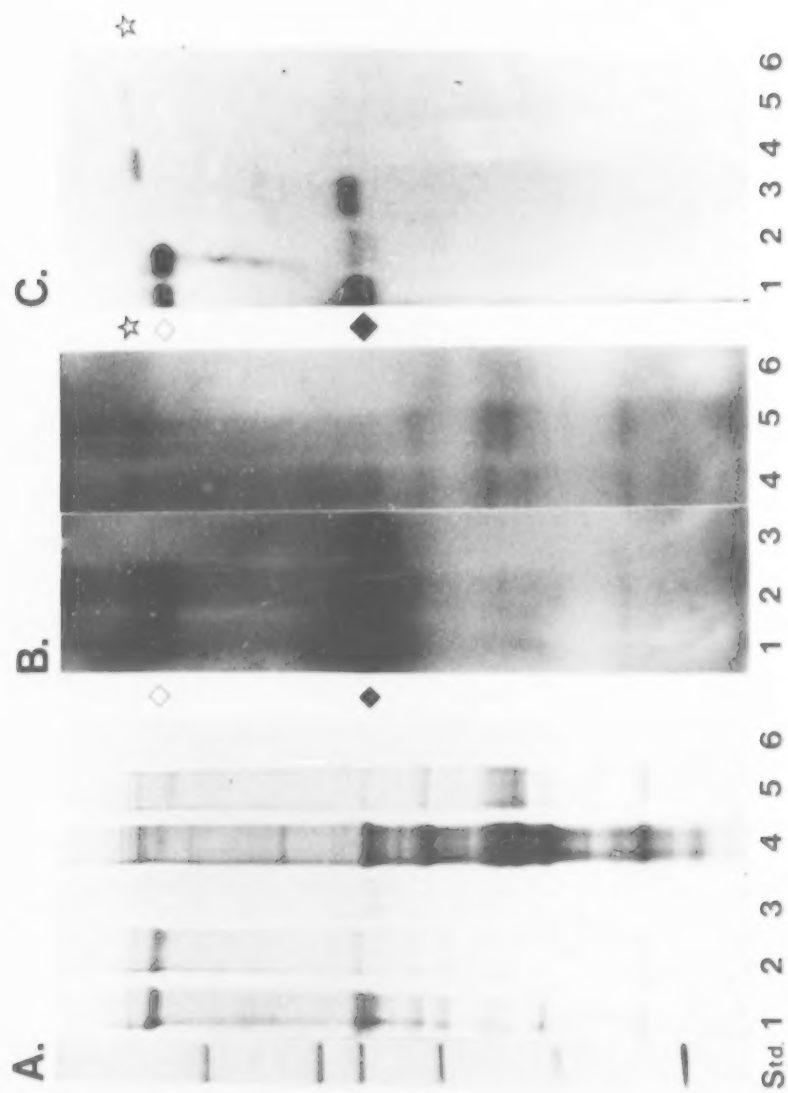


FIGURE VI 3

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED CHICKEN OOCYTE LRP

Avian apo B (10 μ g; *lane 1*), Triton X-100 solubilized oocyte membrane extract (35 μ g of protein; *lane 2*), and immunoaffinity purified chicken oocyte LRP (5 μ g; *lane 3*) were subjected to electrophoresis on a 3-12% SDS-polyacrylamide gradient gel under non-reducing conditions, followed by staining with Coomassie Blue.



FIGURE VI 4

LIGAND BLOTTING OF PURIFIED CHICKEN OOCYTE LRP

After electrophoretic separation in the absence of reducing agent and transfer to nitrocellulose, each strip containing 1.0 μ g of immunoaffinity purified material was incubated for 2 hr in buffer with the following additions: *Lanes: A-C*, ^{125} I-labelled VLDL at 2.4 μ g/ml (145 cpm/ng); *D-F*, ^{125} I-labelled VTG at 2.4 μ g/ml (478 cpm/ng); *B* and *F*, for competition, VLDL at 0.24 mg/ml was added; *C* and *E*, VTG at 0.24 mg/ml was added. The autoradiograph was exposed for 16 hr (*A-C*) or 6 hr (*D-F*) at -70° C.

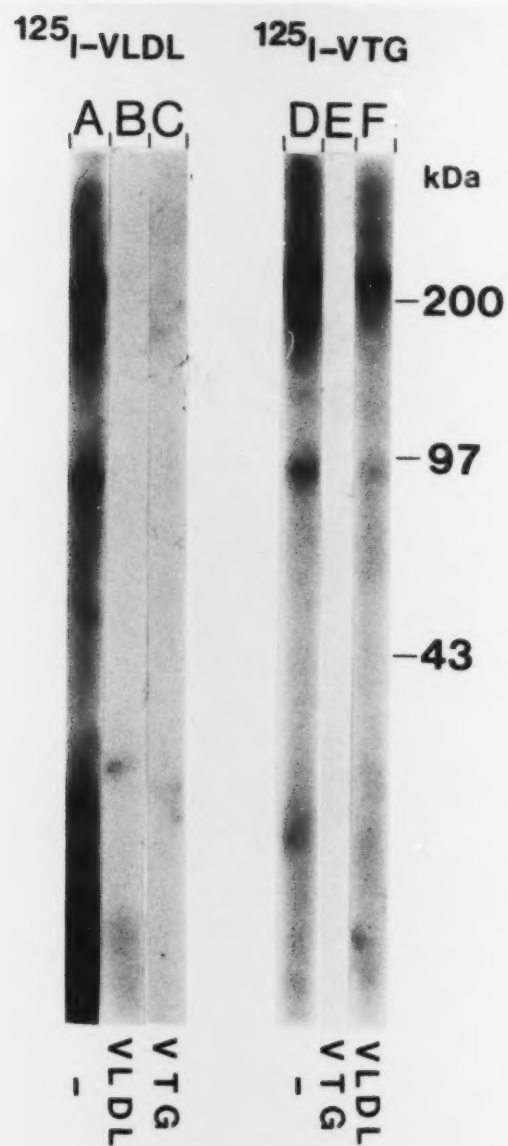


FIGURE VI 5

STRUCTURAL SIMILARITY BETWEEN HUMAN LRP AND CHICKEN 380 kDa PROTEIN

The amino acid sequences of three tryptic fragments of the oocyte-specific protein from chicken (Chi. ooc LRP) are aligned with homologous sequences in human LRP [166]. Numbers refer to the position of the amino terminal residue of each fragment in mature human LRP [166]. Dashes indicate spaces introduced to optimize alignment. Identical residues, or residues conforming to the consensus sequence for the longest of the three peptides (bottom), are boxed in with a thick line, and conservative replacements with a thin line, respectively. "X" denotes an unidentified residue. In the consensus sequence, "Ø" refers to a hydrophobic residue and "+" to a positively charged amino acid.

FIGURE VI 6

SATURATION CURVE AND SCATCHARD PLOT FOR THE BINDING OF CHICKEN ^{125}I -VLDL TO THE PURIFIED CHICKEN OOCYTE LRP

(A) Each assay tube contained the standard assay mixture (100 μl) with 6.2 μg of precipitated immunoaffinity purified chicken oocyte LRP and the indicated concentration of radiolabelled ligand in the presence (■) or absence (●) of 3 mg/ml unlabelled VLDL. The specific activity of the ^{125}I -VLDL was 365 cpm/ng. The amount of receptor-bound radiolabelled ligand was determined as described in Chapter II.O. High affinity binding (□) was calculated by subtracting non-specific binding (■) from total binding (●). Each data point represents the average of duplicate determinations.

(B) Binding data were analyzed by the method of Scatchard [223]. The ratio bound/free is the amount of bound ^{125}I -VLDL (μg of protein) divided by the amount of unbound protein in the reaction mixture (μg of protein).

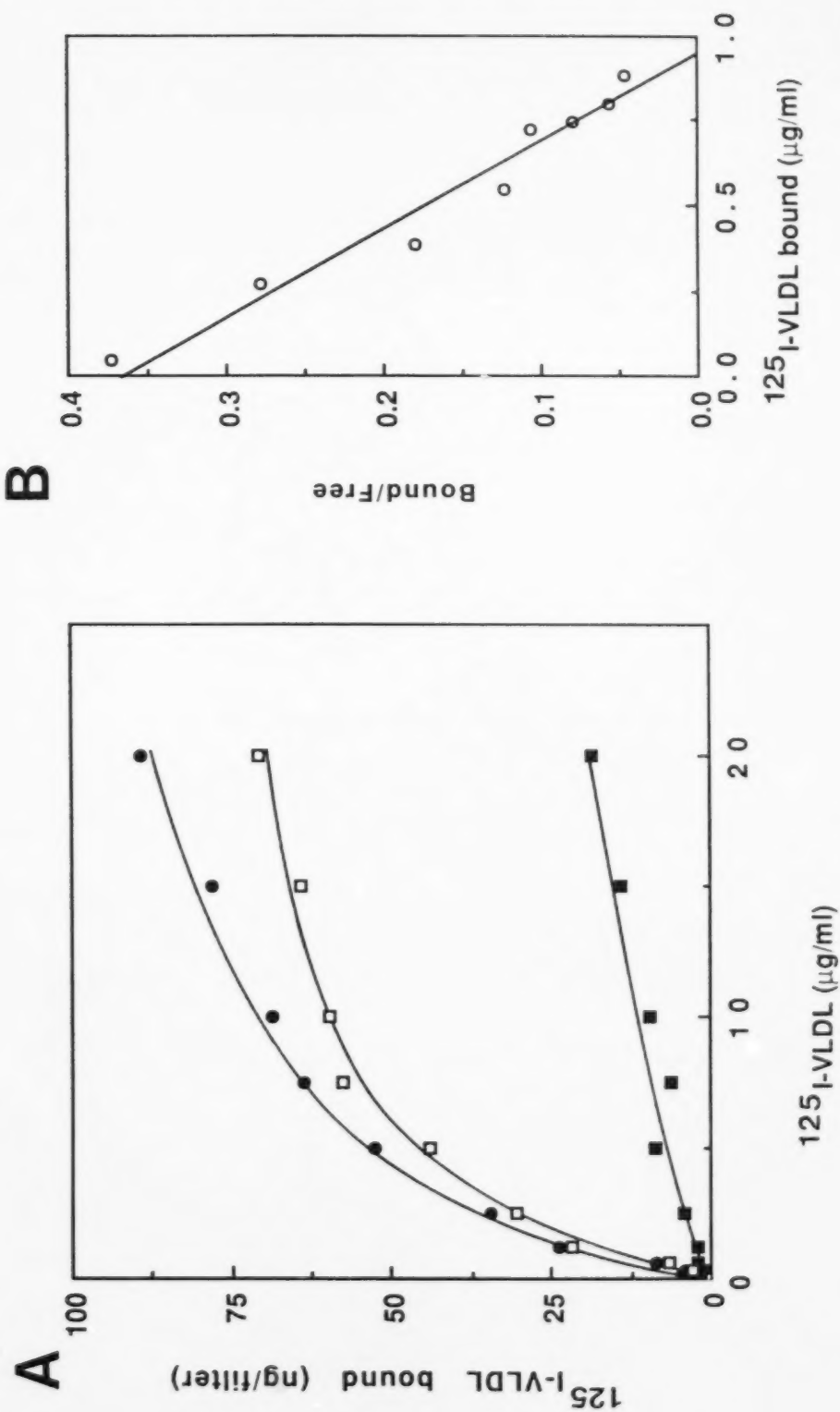


FIGURE VI 7

SATURATION CURVE AND SCATCHARD PLOT FOR THE BINDING OF CHICKEN ^{125}I -LDL TO PURIFIED CHICKEN OOCYTE LRP

(A) Each assay tube contained the standard assay mixture (100 μl) with 5.6 μg of precipitated immunoaffinity purified chicken oocyte LRP and the indicated concentration of radiolabelled ligand in the presence (■) or absence (●) of 6.0 mg/ml unlabelled LDL. The specific activity of the ^{125}I -LDL was 129 cpm/ng. The amount of receptor-bound radiolabelled ligand was determined as described in Chapter II.O. High affinity binding (□) was calculated by subtracting non-specific binding (■) from total binding (●). Each data point represents the average of duplicate determinations.

(B) Binding data were analyzed by the method of Scatchard [223]. The ratio bound/free is the amount of bound ^{125}I -LDL (μg of protein) divided by the amount of unbound protein in the reaction mixture (μg of protein).

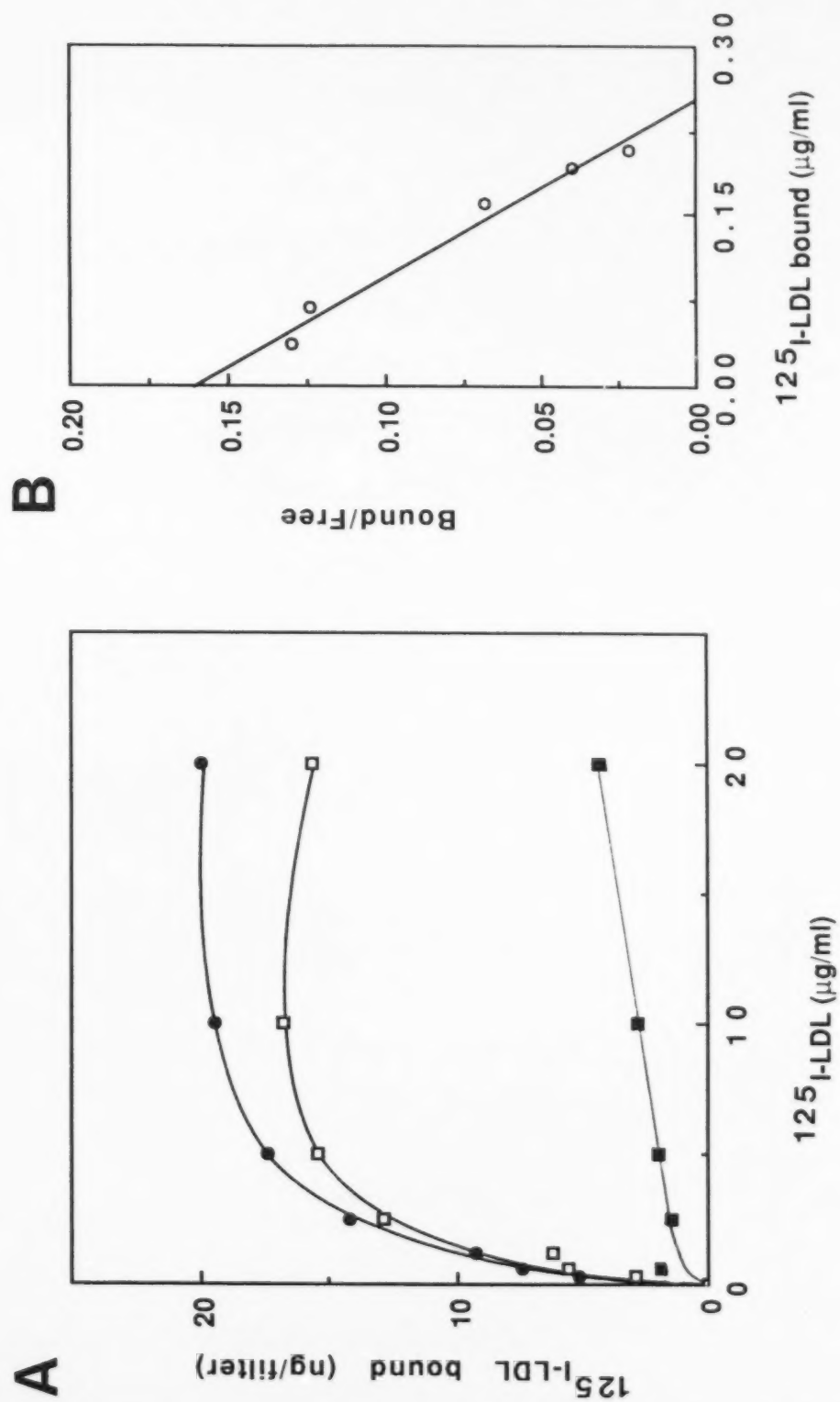


FIGURE VI 8

SATURATION CURVE AND SCATCHARD PLOT FOR THE BINDING OF CHICKEN ^{125}I -VITELLOGENIN TO PURIFIED CHICKEN OOCYTE LRP

(A) Each assay tube contained the standard assay mixture (100 μl) with 8.9 μg of precipitated immunoaffinity purified chicken oocyte LRP and the indicated concentrations of radiolabelled ligand in the presence (■) or absence (●) of 10 mg/ml suramin. The specific activity of the ^{125}I -VTG was 116 cpm/ng. The amount of receptor-bound radiolabelled ligand was determined as described in Chapter II.O. High affinity binding (□) was calculated by subtracting non-specific binding (■) from total binding (●). Each data point represent the average of duplicate determinations.

(B) Binding data were analyzed by the method of Scatchard [223]. The ratio bound/free is the amount of bound ^{125}I -VTG (μg of protein) divided by the amount of unbound protein in the reaction mixture (μg of protein).

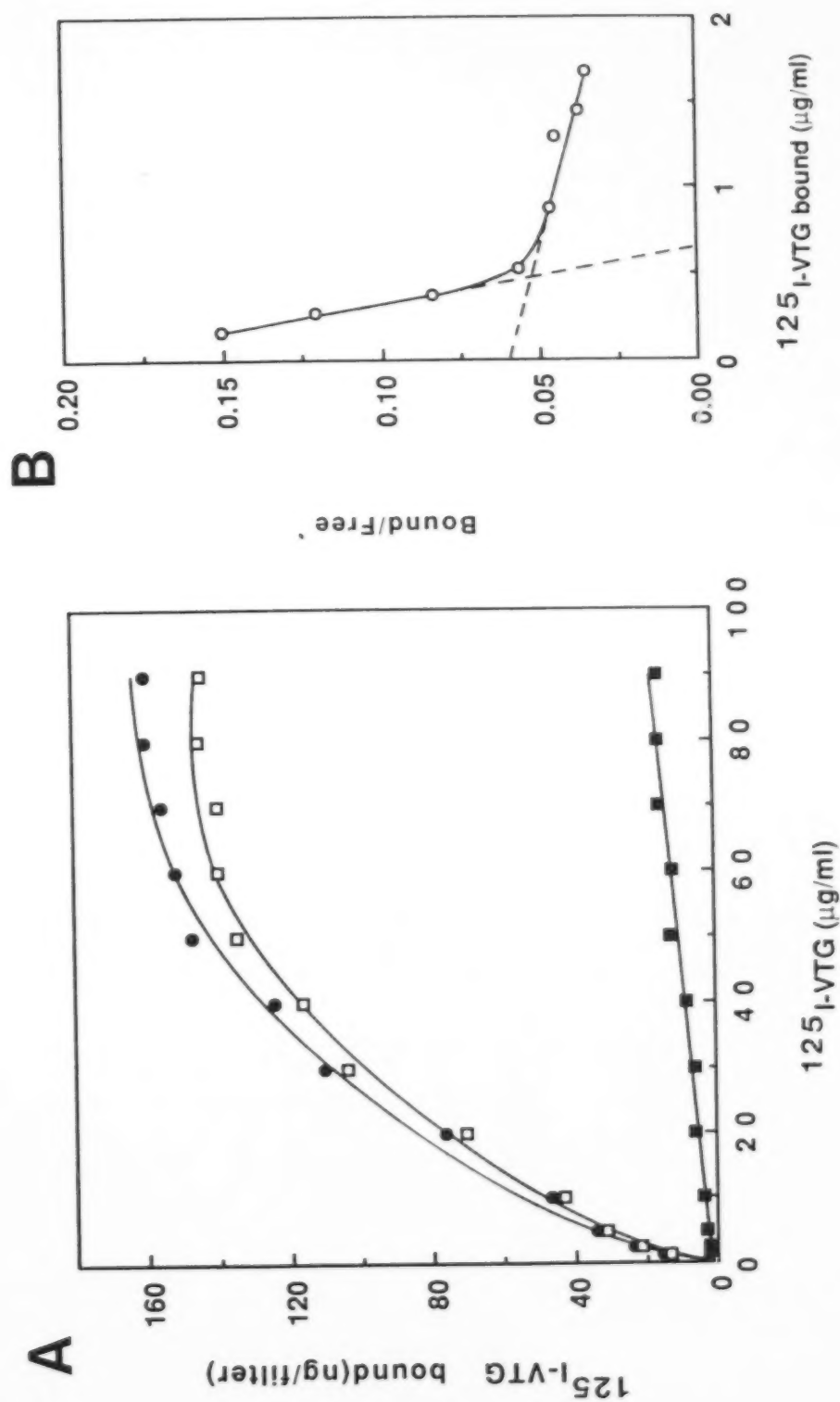


FIGURE VI 9

EFFECT OF UNLABELLED LIGANDS ON THE BINDING OF CHICKEN ^{125}I -VLDL TO PURIFIED CHICKEN OOCYTE LRP

Each assay tube contained the standard assay mixture (100 μl) with 7.0 μg of precipitated immunoaffinity purified chicken oocyte LRP and 10 $\mu\text{g}/\text{ml}$ ^{125}I -VLDL (17 cpm/ng) in the presence of unlabelled laying hen VLDL (○), reductively methylated VLDL (●), laying hen VTG (■), or rooster HDL (◇) at the indicated concentrations. The amount of receptor-bound radiolabelled ligand was determined by filtration as described in Chapter II.O. The 100%-of-control value was 20.7 μg VLDL bound/mg protein.

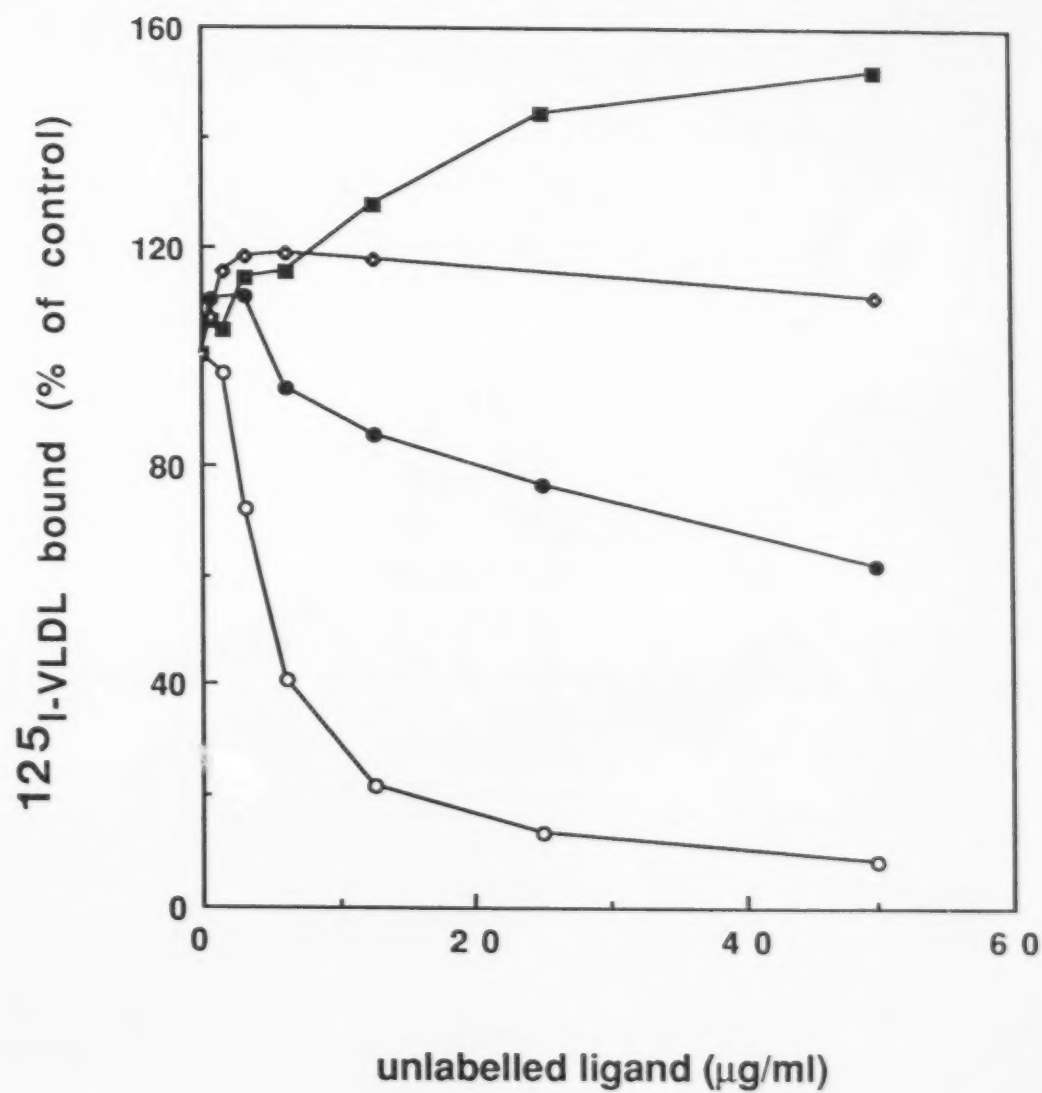


FIGURE VI 10

EFFECT OF UNLABELLED LIGANDS ON THE BINDING OF CHICKEN ¹²⁵I-VITELLOGENIN TO PURIFIED CHICKEN OOCYTE LRP

Each assay tube contained the standard assay mixture (100 μ l) with 8.9 μ g of precipitated immunoaffinity purified chicken oocyte LRP and 10 μ g/ml laying hen ¹²⁵I-VTG (329 cpm/ng) in the presence of unlabelled laying hen VTG ($\square$), reductively methylated VTG ($\blacksquare$), laying hen VLDL ($\circ$), or rooster HDL ($\diamond$) at the indicated concentrations. The amount of receptor-bound radiolabelled ligand was determined by filtration as described in Chapter II.O. The 100%-of-control value was 7.6 μ g VTG bound/mg protein.

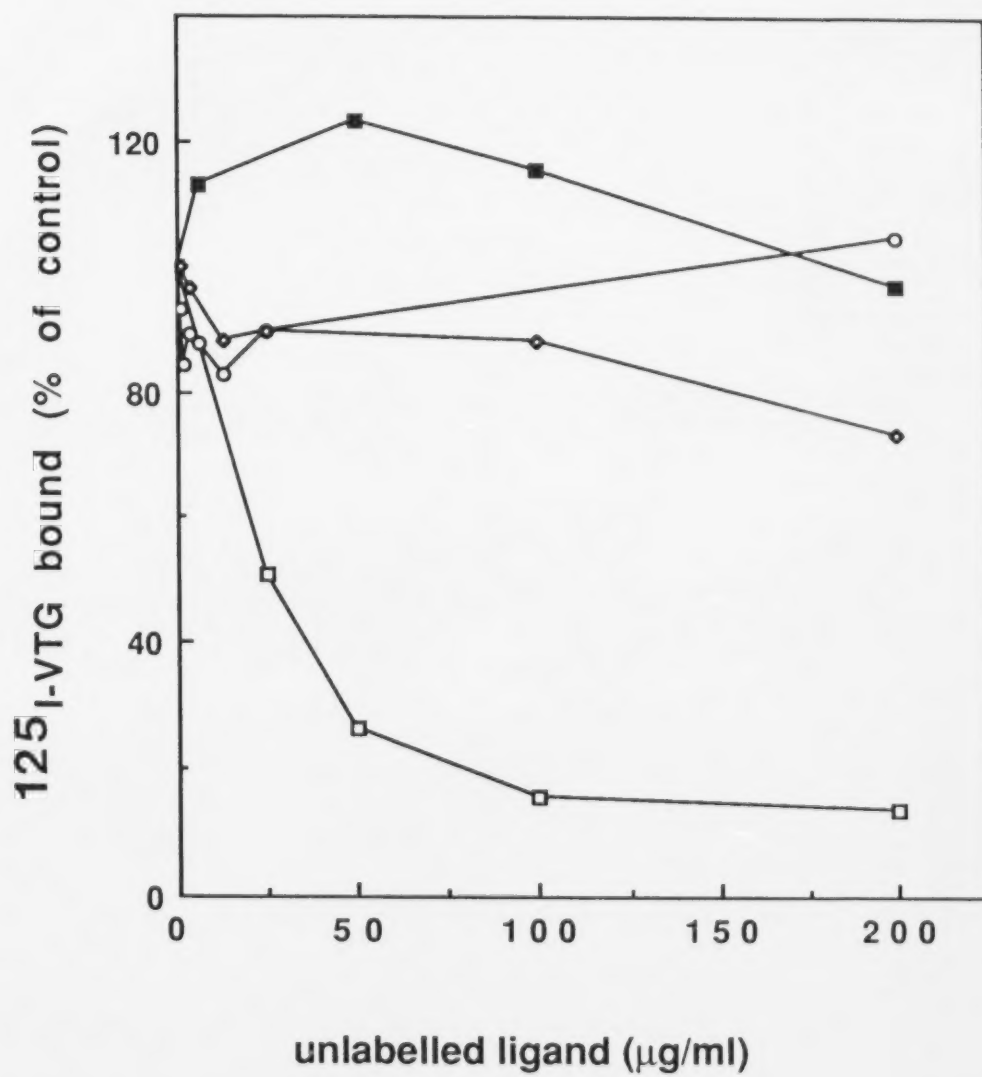
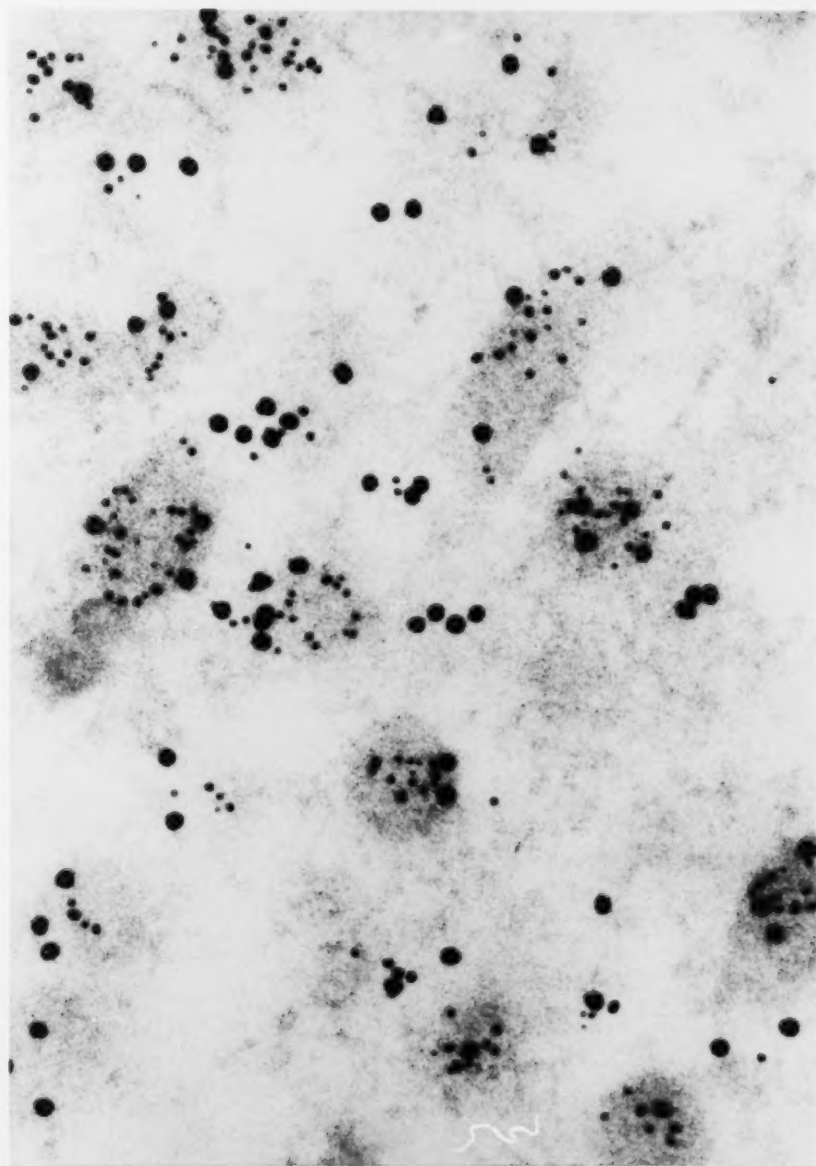


FIGURE VI 11

CO-IMMUNOLOCALIZATION OF THE 95 kDa RECEPTOR AND THE 380 kDa LRP

A section from a 2 mm oocyte near the plasma membrane was selected. One side of the grid was incubated with affinity-purified anti-oocyte receptor IgG followed by 15 nm Protein A-colloidal gold particles. The other side of the grid was incubated with affinity-purified anti-oocyte LRP IgG followed by 5 nm Protein A-colloidal gold.



CHAPTER VII

GENERAL DISCUSSION

The rapid growth of the avian oocyte can be attributed to the uptake of a defined group of serum proteins. The lipid requirements of the developing embryo are in large part met by the uptake of a cholesterol- and triglyceride-rich particle, VLDL, and a complex lipophosphoglycoprotein, VTG. Previous morphological studies illustrated the architecture of the oocyte follicle and suggested that receptor mediated uptake occurred [176]. Lipoprotein-like particles were found localized in coated pits of oocyte membranes and coated vesicles were isolated from within the oocyte [176]. In addition, it was shown that LDL could bind to isolated granulosa cell sheets, suggesting that apo B mediated lipoprotein binding [18]. Independent investigations had described an oocyte VLDL receptor from binding experiments, however, no further characterization of the receptor appeared in the literature [179],[180].

The VLDL receptor was initially isolated as a 95 kDa protein. The receptor was readily solubilized from oocyte membranes using a number of non-ionic detergents. A specific, saturable, VLDL binding activity was present in detergent solubilized oocyte membranes. Solid phase binding experiments and ligand blotting illustrated that a 95 kDa protein was responsible for VLDL binding. Cross-species reactivity was first demonstrated as avian lipoproteins bound to the mammalian LDL receptor. The importance of lipoprotein transport is documented by the evolutionary conservation of lipoprotein ligands and receptors. The 95 kDa receptor functions as a multifunctional transport protein as *in vitro* binding of the apo B-containing lipoproteins, VLDL, LDL, as well as vitellogenin has been demonstrated. Dr. Shen in the laboratory has illustrated that activated α_2 -macroglobulin binds to the 95 kDa receptor in ligand blots [252]. Scatchard analysis of saturation curves and competitive binding studies illustrate that VTG and VLDL may bind to the same site on the receptor. Steric hindrance may not allow VLDL access to the VTG binding site, which may be a smaller structure of the apo B binding site. There is precedence for this assessment as the mammalian LDL receptor cysteine-rich repeat 5 is required for apo E binding, whereas repeats 2-7 are obligatory for apo B binding [152].

Dr. Steyrer in the laboratory has shown that the 95 kDa receptor binds mammalian apo E with high affinity [203]. Interestingly, the avian receptor was unable to discriminate between apo E3/3 which displays normal binding to the human LDL receptor, and apo E2/2 which does not bind to the human receptor and results in Type III hyperlipoproteinemia [121]. The structural basis for the binding of apo E2/2 to the 95 kDa receptor remains to be identified.

The oocyte 95 kDa receptor appears to be more closely related to the mammalian LDL receptor than to its somatic counterpart, the 130 kDa LDL receptor, as a polyclonal antibody raised against the bovine LDL receptor cross-reacts with the avian 95 kDa receptor, but does not recognize the avian LDL receptor expressed in fibroblasts or granulosa cells [228]. It is not clear why this antibody does not react with the avian somatic LDL receptor, however, it does recognize a 110 kDa protein which is present in granulosa cell culture after cells have been exposed to sterols or LDL. This membrane protein is unable to bind apo B-containing lipoproteins and appears in a time dependent fashion after cells have been incubated with sterols in conjunction with a concomitant decrease in the amount of 130 kDa LDL receptor. It is thought that this protein represents an intermediate in the degradation pathway for the somatic LDL receptor, but this has not been tested further [229].

The oocyte 95 kDa VLDL/VTG receptor contains both N- and O-linked carbohydrate. However, the corresponding shift from 120 to 160 kDa during biosynthesis of the mammalian LDL receptor does not occur with the isolated oocyte receptor. Only a small amount of N- and O-linked carbohydrate is present on the 95 kDa receptor, as evidenced by endoglycosidase digestions, which may suggest that the O-linked carbohydrate domain of the LDL receptor may be absent in the oocyte 95 kDa receptor. Once avian oocytes are successfully grown in culture, biosynthetic studies could address this question further.

Another lipoprotein binding protein was identified from this study. Sequence information from tryptic peptides verified that the 380 kDa peripheral membrane protein was an oocyte LDL receptor-related protein. This protein displayed binding of the identical ligands namely, VLDL, LDL and VTG, with similar affinity as to the 95 kDa receptor (Table VII 1). Scatchard analysis revealed that VLDL and LDL bind to LRP-380 with a single class of binding sites, whereas VTG shows both high and low affinity binding sites. K_d values for ligand binding to both the oocyte 95 kDa receptor and oocyte LRP are in the nM range; LDL shows a 3-fold higher affinity for LRP-380. Independent binding sites for apo B and VTG are found on LRP-380, since no ligand cross-competition is observed. As above, Dr. Shen has illustrated in ligand blotting experiments binding of activated α_2 -macroglobulin to oocyte LRP [252].

The co-operation between the oocyte 95 kDa receptor and oocyte LRP in receptor mediated oocyte transport is unclear. Both proteins are found in vesicular fractions in ultrastructural immunolocalization experiments. Perhaps the oocyte LRP is expressed at certain stages during oocyte growth to help facilitate the massive import of protein into the growing oocyte. Alternatively, *in vivo* it may selectively bind only one particular ligand, say activated α_2 -macroglobulin. LRP-380 could act as an autocrine factor and stimulate granulosa cell growth in the oocyte follicle or within a fertilized oocyte. It is known that not only does α_2 -macroglobulin become activated through binding of proteases, but growth factors such as transforming growth factor- β [253], platelet-derived growth factor [254] and basic fibroblast growth factor [255] bind to this serum protein. α_2 -macroglobulin could act to transport growth factors necessary for embryo development into the oocyte via the 95 kDa receptor or oocyte LRP. Alternatively, LRP-380 could play a role in ligand delivery within the oocyte to yolk granules or yolk platelets.

Human somatic LRP exists as a 600 kDa precursor which is processed at the sequence RHRR into two chains, the extracellular domain termed LRP-515 and the membrane bound light chain, termed LRP-85. The present results indicate that oocyte

TABLE VII 1

KINETIC PARAMETERS FOR LIGAND BINDING TO OOCYTE RECEPTORS

LIGAND	K _d (nM)	
	95 kDa RECEPTOR	380 kDa LRP
VLDL	2.7	2.7
LDL	9.0	3.1
VTG -Site I	6.4	8.8
-Site II		129

LRP-380 is a processed form of a larger, yet unidentified precursor; in addition, the biochemical tools to identify the corresponding oocyte LRP-85 are not yet available.

The restricted ovulator (R/O) hen does not express functional 95 kDa VLDL/VTG receptor and is unable to lay. As shown by Dr. Nimpf in the laboratory, LRP-380 is found in follicle membranes from R/O hens, illustrating that expression of oocyte LRP alone is insufficient to mediate delivery of ligands during the rapid growth phase of the oocyte [232]. It is evident that the 95 kDa VLDL/VTG receptor plays a vital role in the uptake of a number of proteins into the oocyte. Elucidation of the exact mechanism, role and interrelationships of the 95 kDa receptor and LRP-380 will be the subject of future investigations. Isolation of full length cDNA clones for the proteins will be an important step in determining structure-function relationships responsible for receptor-mediated oocyte growth.

The receptor binding sequence of apo E has been localized to a region where a large number of positive charges are located. Corresponding sequences have been discovered in human apo B (see Figure VII 1). VTG is not synthesized in mammalian species, whereas apo E is not found in oviparous species. Two strings of basic residues are found in the lipovitellin-I moiety of VTG which could act to mediate VTG binding to the 95 kDa receptor. The appearance of apo E in mammalian species may suggest that it has evolved as a mammalian counterpart to VTG. The receptor binding of apo E, apo B and VTG is blocked by reductive methylation. Although corresponding sequence information for chicken apo B is currently unavailable, receptor binding epitopes are probably highly conserved. The binding of VLDL and VTG is mediated via basic residues that interact with SDE sequences within the oocyte 95 kDa receptor [152] and oocyte LRP.

Specific proteolysis occurs accompanying the receptor mediated uptake of VLDL and VTG by cathepsin D [201]. Proteolytic cleavage of apo B does not affect native receptor binding sites as the VLDL fraction isolated from yolk displays normal binding to the 95 kDa receptor. Furthermore, reverse ligand blotting experiments have shown that the

95 kDa receptor interacts specifically with two fragments of 56 and 79 kDa from yolk apo B and 30, 78 and 110 kDa lipovitellin products.

During the course of this research several new members of the LDL receptor family were described that allows one to examine the development of this group of membrane proteins in an evolutionary context. Analysis of LDL receptor family domain structure (Figure 14), suggests that LRP has evolved as a cassette duplication of the LDL receptor. However, lower oviparous species require VTG uptake for embryo survival. Candidate oocyte receptors have been identified by Stefano Stifani in *Xenopus* [58] and coho salmon [199]. It is not known if the oocyte receptor is present in lower vertebrates. Somatic LRP also functions as the receptor for α_2 -macroglobulin [174], [250] but shows binding of VTG as well [252]. An LRP has been identified in *C. elegans* and bears striking homology to human LRP. VTG and α_2 -macroglobulin have been identified in lower invertebrates. Therefore, LRP could have evolved as a primordial multifunctional transport receptor. The oocyte VLDL/VTG receptor may have developed some time later, to satisfy the massive lipid requirement of the higher vertebrate embryo. The somatic 130 kDa receptor has evolved as a specialized receptor that mediates the transport of apo B containing lipoproteins into somatic tissues.

This study has illustrated a dichotomy in the LDL receptor family. A functional 130 kDa LDL receptor is expressed in somatic tissues whereas a multifunctional 95 kDa receptor is found in the oocyte membrane. Somatic tissues also express a LRP-600 which is proteolytically processed at the sequence RHRR into LRP-515 and LRP-85. A processed LRP counterpart has been identified in the oocyte membrane, a 380 kDa protein. However, the intact precursor and membrane-bound oocyte LRP-85 remain to be identified. The 95 kDa receptor and LRP-380 mediate the transport of a number of ligands into the oocyte. Future investigations will address the individual role that each protein plays in receptor mediated oocyte growth. Due to the differential tissue expression of LDL

receptors, the avian system may prove to be the most informative in delineating the interrelationships of the various members of the LDL receptor family.

FIGURE VII 1

SEQUENCE COMPARISON OF LIPOPROTEIN RECEPTOR LIGANDS

The sequence of the receptor binding domain of apo E [135], [136] is aligned with homologous sequences from human apo B [96] and chicken VTG [50]. Numbers refer to the position of the amino terminal residue of each fragment. Basic residues appear in bold face.

Human apo E

143 H L R K L R K R L L R

Human apo B

3147 K A Q Y Y K N K H R H

Human apo B

3359 T T R R T R K R G L K

Chicken VTG

1079 K L K R I L

Chicken VTG

493 L K R I L K

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